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RENAL PLASMA FLOW AND SODIUM REABSORPTION AND EXCRETION IN CONGESTIVE HEART FAILURE¹

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Warren and Stead's (1) contention that disturbed renal function secondary to a diminished cardiac output is responsible for the following series of events—salt and water retention, increased blood and extracellular fluid volume, rise in venous pressure, edema—seemed to offer a rational explanation for some of the clinically observed phenomena. We therefore initiated a series of studies on patients with chronic congestive heart failure, using the clearance techniques of Smith and associates (2), in order to evaluate the relationship between the decreased sodium excretion in heart failure (3, 4) and renal blood flow, to determine the nature of the disturbance in renal function and the relationship, if any, between the altered renal dynamics and sodium retention. We later attempted to define some of the variables involved in the tubular transfer system for sodium as it obtains in the normal and in the cardiac patient. Since our studies began, Merrill (5) reported that the renal plasma flow was reduced to as little as 20 per cent and the filtration rate to 33 per cent of normal in chronic congestive failure. We have been able to confirm his findings of a decreased sodium excretion rate due to a diminished load presented to the tubules for reabsorption, and not to enhanced tubular reabsorption as suggested by earlier workers (3).

EXPERIMENTAL PROCEDURE

Patients with advanced chronic congestive failure due predominantly to rheumatic heart disease were the subjects. All had variable amounts of edema at rest. Members of the resident house staff and patients without heart failure or renal disease served as controls.

The subjects were brought to the laboratory in a post-absorptive state. Each patient was given 300 to 600 cc. of water about 30 to 60 minutes before the test period.

Five controls were maintained on a special cardiac salt-poor diet (about 1.3 grams of sodium chloride daily), for 4 to 5 days before the studies were made. Most of the patients with congestive heart failure were maintained on the same diet (strict metabolic control was not attempted) and, in addition, all were taking digitalis. Except for 1 cardiac who, by error, received mercurpurin 48 hours before the test period, none had received mercurials or other diuretics for at least 72 hours prior to the clearance studies.

The effective renal plasma flow was measured with sodium para-aminohippurate³ and the glomerular filtration rate with mannitol, as recommended by the New York University group (6, 7). Priming solutions contained 55 to 80 cc. of 25 per cent mannitol and 2 to 5 cc. of 20 per cent para-aminohippurate, depending on the estimated size of the extracellular fluid compartment. Sustaining solutions were given at the rate of 4 cc. per minute and consisted of 4 to 5 per cent mannitol and 0.10 to 0.25 per cent para-aminohippurate made up in distilled water instead of saline. In 2 experiments, 4 per cent saline was used as the diluent. Three or more 20- to 30-minute urine collection periods were carried out with an indwelling 6-holed catheter, the bladder being washed out with 10 to 20 cc. distilled water. Venous blood samples were obtained at the beginning and end of each urine collection period and the concentration of the various substrates were plotted on semilogarithmic paper against time. Under our test conditions, the plasma mannitol concentration curve usually had a rising slope and the para-aminohippurate curve a falling slope. The mean concentration was obtained by interpolation to the middle of each period. The data for the clearance values are the averages for the 3 or more collection periods. They are all corrected to a body surface area of 1.73 sq. m. We have used the body surface estimated from the weight which is ideal for a subject of given height, age, and sex rather than the actual surface estimated from the height and weight at the time of observation (8). In several instances, there was an appreciable correction factor in spite of edema because many of these patients had lost considerable body tissue secondary to their long-standing congestive failure. Had these correction factors not been applied, many of the clearance values would have been appreciably lower than are here reported. The chemical methods used are described in detail by Smith *et al.* (6) and Goldring and Chasis (9).

¹ This study was aided by a grant from the Martha M. Hall Foundation and the Committee on Scientific Research of the American Medical Association.

² Martha M. Hall Foundation Fellow in Medicine.

³ Liberal quantities of sodium para-aminohippurate and mannitol were supplied by Dr. J. Wm. Crosson of Sharpe and Dohme.

TABLE I

Summary of data
A. Patients with congestive failure

Patient	Age	Sex	Venous pressure	Renal plasma flow	Filtration rate	Filtration fraction	Urine flow	Hematocrit	Blood volume	*Deviation from predicted normal volume	Plasma Protein		
											Albumin	Globulin	Total circulating
			cm. water	cc. per min. per 1.73 sq.m.		per cent	cc. per min.	per cent cells	cc.	per cent	grams per cent	grams per cent	grams
E. F.	37	M.	25	166	52	31.3	1.2	32.3	7020	+35	3.6	2.9	308
P. B.	23	F.	26	235	76	32.3	2.2	35.7	6090	+52	5.0	3.3	324
			25	225	82	36.5	2.6	41.6	7050	+76	4.8	3.3	334
A. G.	30	F.	11	225	58	25.8	2.4	30.9	5900	+47	5.7	3.1	358
			19	181	66	36.5	2.1	34.6	7220	+80	5.1	2.8	372
L. C.	16	F.	20	266	81	30.4	3.0	38.1	3950	§	4.8	2.4	176
R. D.	53	M.	32	253	83	32.8	1.6	36.0	7160	+28	4.9	2.9	360
E. S.	56	M.	20	129	54	41.8	1.4	45.0	9050	+74	3.8	2.8	328
I. F.	46	F.	13.5	218	65	29.8	2.3	38.3	3440	§	4.7	2.7	141
†H. Z.	23	M.	18	138	82	59.4	2.0	43.1	6700	+13	3.7	2.2	225
S. O.	41	F.	23	172	60	34.9	2.1	40.0	4730	+17	2.9	4.7	215
O. W.	37	F.	25	138	54	39.1	2.3	39.6	5040	+40	3.9	4.0	232
A. M.	40	M.	13	150	58	38.7	3.1	43.2	5380	+ 2.0	5.3	3.6	272
B. G.	24	F.	30	175	71	40.6	2.7	42.6	4350	+21	4.4	2.7	177
E. G.	31	F.	17	175	74	42.3	2.9	45.0	6530	+70	3.6	2.5	219
			24	106	70	66.0	1.6	43.1	7400	+95	2.5	1.4	164
†S. L.	46	F.	17	180	32	17.8	1.6	46.1	5370	+68	4.7	1.5	180
†G. B.	42	M.	10.5	315	86	27.3	3.4	51.0	6260	+12			
W. S.	40	M.	21	188	66	35.1	3.8	54.5	6930	+24	3.9	1.6	173
mean:				191.5	66.8	37.8							
σ				54.4	13.2	9.9							

B. Normal controls and cardiac patients without congestive failure

B. D.	22	M.	7	600	96	16.0	4.8	44.5	5730	- 1.2			
R. M.	29	M.		801	133	16.6	5.5						
I. B.	23	F.	6	700	102	14.6	3.6	40.5	3920	- 2.0			
I. R.	30	M.		575	94	16.4	3.0						
J. P.	28	M.		723	98	13.6	2.2						
I. L.	30	M.	7	778	110	14.2	5.7						
L. B.	35	F.	5	624	99	15.9	7.8	41	3990	+ 5.0			
E. G.	45	F.	6	570	87	15.3	3.1	38.6	3760	§			
N. L.	34	M.	8	655	117	17.9	4.4	43.3	4860	-10			
E. S.	20	M.	9	560	97	17.3	3.6	44.1	4570	- 8.6			
†C. McK.	42	F.	8	546	98	18.0	4.7						
†F. M.	24	M.	6	565	125	22.1	4.6	45.0	4930	+ 0.61			
†A. M.	25	M.	5	576	97	16.8	4.8	48.0	5850	- 0.85			
H. K.	40	M.	5	516	91	17.6	3.1	49.0	4260	- 2.4			
mean:				627	103	16.6							
σ				86.6	12.8	2.0							

* Predicted blood volumes were based on height from the data of Gibson and Evans (15.).

† All the congestive failure patients had rheumatic heart disease except H. Z., S. L., and G. B., who had healed subacute bacterial endocarditis, amyloid heart disease, and hypertensive heart disease, respectively.

‡ Non-failure cardiac patients.

§ Insufficient data for volume prediction on such short patients.

Sodium determinations were made by uranyl zinc acetate precipitation following a preliminary dry ashing of the sera and of the urine when the concentration of sodium in the latter was less than 0.1 mgm. per cc., as recommended by Butler and Tuthill (10). Total serum protein nitrogen was determined manometrically after the usual micro-Kjeldahl digestion (11). Albumin and glob-

ulin separation was carried out according to the method described by Campbell and Hanna (12). Plasma volume was determined with the blue dye T-1824, the optical density being measured with the photoelectric colorimeter. In preliminary experiments, we determined the plasma volumes in patients with congestive failure from extrapolation of a time-concentration curve and from a single

SODIUM REABSORPTION IN CONGESTIVE HEART FAILURE

TABLE II

Sodium clearance data; corrected to 1.73 sq. m.: average of 3 periods

A. Patients with congestive heart failure

Patient	Glomerular filtration rate	Sodium					Remarks
		Serum conc.	Filtered*	Excreted	Reabsorbed	Reabsorbed per 100 cc. glomerular filtrate	
	cc./min.	mM/L.	mM/min.	mM/min.	mM/min.	mM	
E. F.	52	131	6.81	.0052	6.80	13.1	
P. B.	76	150	11.4	.109	11.3	14.9	On regular diet
	82	137	11.2	.107	11.1	13.5	On regular diet
A. G.	58	135	7.84	.135	7.70	13.3	On regular diet
	66	137	9.04	.107	8.93	13.5	On regular diet
L. C.	81	139	11.3	.143	11.2	13.8	Mercupurin 48 hours before
R. D.	83	124	10.3	.0026	10.3	12.4	
E. S.	54	132	7.13	.0014	7.13	13.2	
I. F.	65	129	8.39	.076	8.31	12.8	
H. Z.	82	134	11.0	.0052	11.0	13.4	
S. O.	60	136	8.16	.0037	8.16	13.6	
O. W.	54	133	7.18	.042	7.14	13.2	
A. M.	58	129	7.48	.129	7.35	12.7	Regaining compensation. On regular diet
B. G.	71	131	9.30	.010	9.29	13.1	
E. G.	70	139	9.74	.0038	9.74	13.9	
	74	141	10.4	.0091	10.4	14.0	
S. L.	32	137	4.38	.085	4.29	13.4	Infusion fluids contained 4 per cent sodium chloride
G. B.	86	141	12.1	.090	12.0	14.0	
W. S.	66	135	8.91	.0065	8.90	13.5	

B. Normal controls and cardiac patients without congestive failure

B. D.	96	135	13.0	.0416	13.0	13.5	Low sodium diet†
R. M.	133	127	16.9	.180	16.7	12.6	
I. B.	102	122	12.4	.185	12.2	12.0	
I. R.	94	135	12.7	.0853	12.6	13.4	Low sodium diet
J. P.	98	134	13.1	.0610	13.0	13.3	Low sodium diet
I. L.	110	138	15.2	.0808	15.1	13.7	Low sodium diet
L. B.	99	134	13.3	.478	12.8	13.0	
E. G.	87	129	11.2	.0788	11.1	12.8	Low sodium diet
N. L.	117	138	16.1	.514	15.6	13.3	Infusion fluids contained 4 per cent sodium chloride
E. S.	97	142	13.8	.365	13.4	13.8	
C. McK.	98	138	13.5	.652	12.8	13.1	
F. M.	125	135	16.9	.398	16.5	13.2	
A. M.	97	138	13.4	.514	12.9	13.3	

* The amount filtered is the product of the plasma concentration and the glomerular filtration rate (mannitol clearance). The amount excreted is the product of the urine concentration and the urine flow. The quantity reabsorbed is the difference between filtered and excreted amounts. See Table I for urine flow.

† Low sodium diet contains about 1.3 grams of NaCl. These subjects were on this diet for 4 to 5 days prior to test period.

serum sample drawn 15 minutes after injection of the dye. The percentage error was 5 per cent or less. Therefore, in later experiments the single dye sample was used to determine the plasma volume using 10-minute samples (13) for normals and 15-minute samples for patients in congestive failure.

The venous pressure was measured with an L-shaped glass manometer filled with distilled water and heparin. The reference point was 5 cm. below the fourth right costochondral junction, with the patient in the supine position.

RESULTS

Judging by the increased blood volume and elevated venous pressure, the degree of heart failure was moderate to severe (Table I). As was to be expected in a random group, with varying degrees of heart failure, there was no significant correlation between these 2 measurements. This was also found by Meneely and Kaltreider (14). A. M. was the only patient with a normal blood

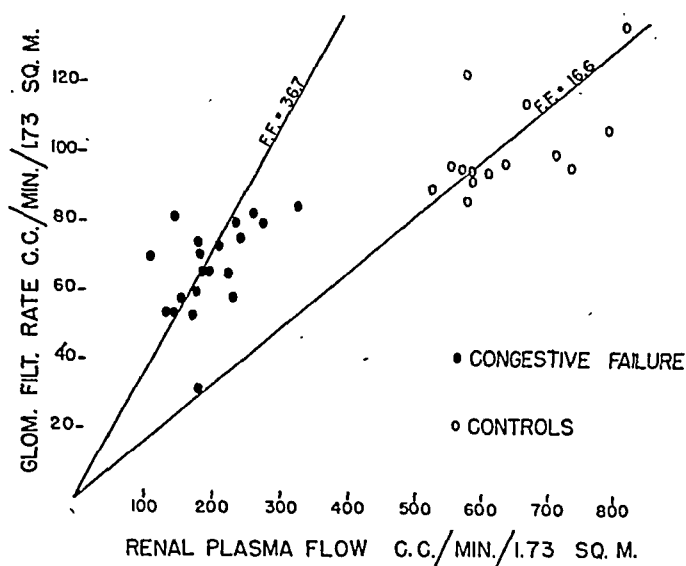


FIG. 1. THE GLOMERULAR FILTRATION RATE IN RELATION TO RENAL PLASMA FLOW IN CONTROLS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE

Each datum represents the average of at least 3 consecutive clearance periods. All patients are included in determining the mean values. The high filtration fraction in congestive heart failure is evident.

volume. This was his first attack of heart failure and he was studied when compensation was almost completely effected; also there was atrophy of the left arm and left leg due to an old hemiplegia.

The data pertaining to the clearance studies are given in Table I. The figures are the averages of 3 or more periods. It will be seen that the cardiac in congestive failure has a mean renal plasma flow of 191.5 ± 54.4 cc. per minute as compared to a mean of 627 ± 86.6 cc. per minute in our group of 14 non-failure control patients. The glomerular filtration rate was 66.8 ± 13.2 cc. per minute in the cardiacs and 103 ± 12.8 cc. per minute in the controls. Thus, in congestive failure, the renal plasma flow is reduced on the average to approximately $\frac{1}{3}$ and the filtration rate to approximately $\frac{2}{3}$ of normal. The ratio of the mannitol and para-aminohippurate clearances or filtration fraction (FF), is considered to be a measure of the fraction of plasma filtered at the glomerulus. Our patients in heart failure have markedly elevated filtration fractions. If we omit 1 case, S. L., with a normal filtration fraction, who at necropsy had primary generalized amyloidosis including the myocardium and renal arterioles, the mean FF in the cardiacs was 37.8 ± 9.9 per

cent. In our controls, which also included both sexes, FF had a mean value of 16.6 ± 2.0 per cent. The differences between the 2 groups are illustrated in Figure 1.

Table II summarizes the sodium clearance data. It is apparent that the patient's sodium intake prior to the test period influences the excretion rate, irrespective of the load that is filtered. Thus, 2 patients who were recovering cardiac compensation, A. G. and A. M., when placed on the routine ward diet (about 10 grams of NaCl), excreted more than 0.1 mM of sodium per minute, whereas 5 of the controls on a low sodium intake (about 1.3 grams of NaCl) averaged 0.07 mM per minute during the test period. However, most of the congestive failure patients on the same limited sodium intake had $\frac{1}{2}$ to $\frac{1}{50}$ the control excretion rates. The decreased sodium excretion of the congestive failure patient was also shown after intravenous infusion of 4 per cent saline given under similar conditions to both a cardiac in failure and to a control patient. The former excreted only 0.085 mM per minute as compared to 0.514 mM per minute for the control, or only about 17 per cent of the normal during the 90-minute observation period.

Despite the variations in the absolute quantities of sodium filtered, reabsorbed, and excreted in the 2 groups, a relatively constant fraction of the sodium in the glomerular filtrate is reabsorbed. This amounts to a mean of 13.3 mM per 100 cc. of glomerular filtrate. Thus, while the cardiac in failure is delivering a smaller load of sodium to his tubules for excretion, his tubular capacity for sodium reabsorption is maintained. This is also illustrated in Figure 2, where it is evident that the rate of sodium reabsorption is a direct function of the glomerular filtration rate (mannitol clearance). It is interesting to note that this linear relationship between filtration rate and reabsorptive capacity has also been found by Pitts and Lotspeich (16, 17) to apply to the anions, bicarbonate and chloride. In their report, they raise the question and prove the point that "functional increases in filtration rate are accompanied by essentially equivalent increases in reabsorptive capacity." We have increased the filtration rate (Table III) in the normals from 100 cc. to 219 cc. maximally by infusing hypertonic sodium chloride

solution (5 and 10 per cent), and in the cardiacs from 62 to 91 cc. by the single intravenous injection of 0.72 gram of aminophyllin and/or 60 mgm. per minute in the infusion mixture. It was similarly found under our experimental conditions

TABLE III

Data illustrating the effect of altering the glomerular filtration rate upon the quantity of sodium reabsorbed

Experiment I was performed on the normal. Experiment II was performed on a patient in severe congestive heart failure. There is no correction for body surface.

Glomerular filtration rate	Urine flow	Sodium				
		Plasma conc.	Filtered*	Excreted	Reabsorbed†	Reabsorbed per 100 cc. glomerular filtrate
cc./min.	cc./min.	mM/L.	mM/min.	mM/min.	mM/min.	mM

Experiment I

Infusion {10 per cent NaCl
1.5 per cent mannitol} 10 cc. per min.

143	7.1	153	20.8	0.983	19.8	13.8
173	14.0	154	25.3	2.29	23.0	13.3
176	18.5	159	26.6	3.01	23.6	13.4
219	24.0	158	32.9	4.13	28.8	13.1

NaCl infusion stopped

182	12.6	155	26.8	2.26	24.5	13.4
151	12.8	155	22.2	2.33	19.9	13.2
128	11.1	155	18.9	1.93	17.0	13.3
119	8.9	154	17.4	1.55	15.9	13.3
111	7.8	152	16.0	1.47	14.5	13.1
102	5.9	149	14.4	1.15	13.3	13.0

Experiment II

Infusion {0.0 per cent NaCl
4 per cent mannitol} 4 cc. per min.

69	1.78	140	9.16	.00492	9.16	13.3
62	1.53	138	8.13	.00311	8.13	13.1
63	1.48	139	8.32	.00259	8.32	13.2

Injection, 0.72 gram aminophyllin intravenously

91	3.76	142	12.3	.0128	12.3	13.5
89	4.56	142.5	12.1	.0212	12.1	13.6
90	4.78	141	12.1	.0247	12.1	13.5

* A constant of 0.95 was used to correct for the Donnan equilibrium.

† In patients with severe congestive failure, the amount of sodium excreted is insignificant in comparison to the filtered load. Hence, the reabsorbed and filtered quantities are identical. We have administered 4.8 per cent sodium chloride in combination with aminophyllin to such patients and have found that the excreted amount of sodium increases to about 0.3 to 0.5 mM per min. Under such circumstances, the amount reabsorbed per 100 cc. glomerular filtrate is greater than when sodium is not administered, but it is always less than the plasma sodium concentration.

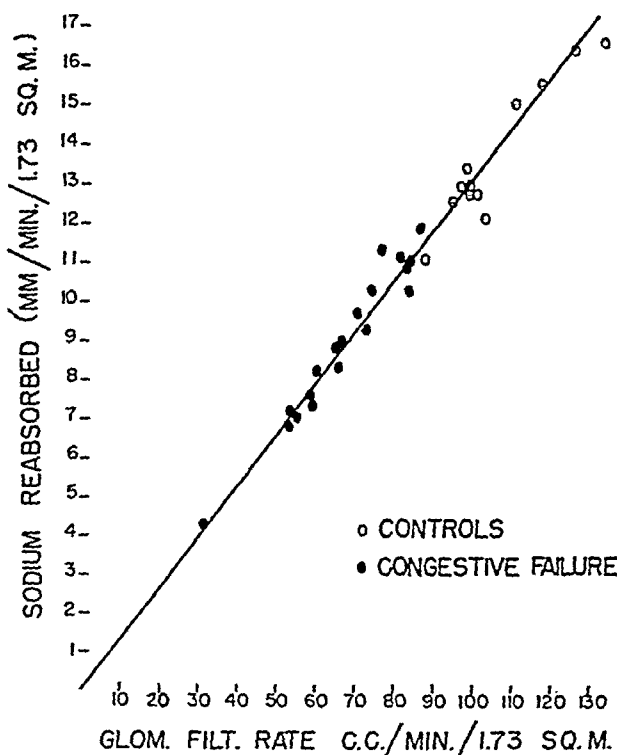


FIG. 2. THE LINEAR RELATIONSHIP BETWEEN THE RATE OF SODIUM REABSORPTION (MILLIMOLS PER MIN.) AND THE GLOMERULAR FILTRATION RATE (CC. PER MIN.)

As in the previous figure each datum is the average of at least 3 clearance periods. The slope of the line is equivalent to 13.3 mM of sodium reabsorbed per 100 cc. of glomerular filtrate. The sodium transfer mechanism in the tubule of the congestive failure patient is normal.

that functional increases in filtration rate are attended by proportional increases in tubular reabsorptive capacity. Hence, when the latter is expressed in millimols reabsorbed from each 100 cc. of glomerular filtrate, a constant quantity is obtained (Figure 3).

From the data in Table I, it is also apparent that there is no relationship between the venous pressure and the reduced renal plasma flow. The increase in total circulating proteins with a slight decrease in concentration due to hemodilution is the usual finding in congestive heart failure (18). Two of our cases, E. G. and S. O., had a low plasma albumin concentration, which we attribute to the deficient protein intake common in the later stages of congestive heart failure. The mean arterial pressure of our subjects was within the normal range. The reduced renal plasma flow was therefore not due to a decrease in driving

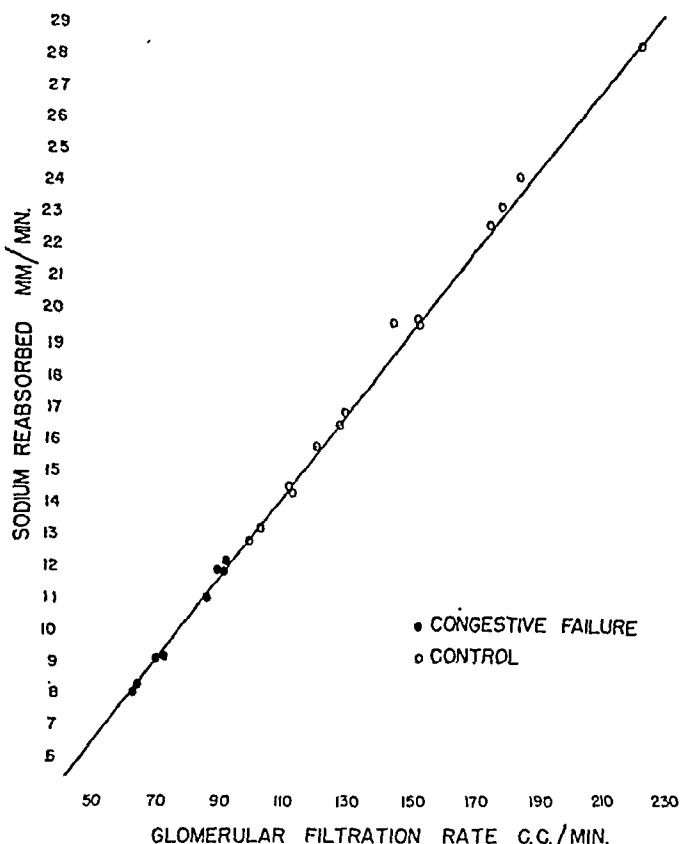


FIG. 3. SHOWING THE EFFECT OF VARYING THE FILTRATION RATE UPON THE AMOUNT OF SODIUM REABSORBED

The constancy of the fraction of sodium reabsorbed is maintained. The filtration rate in the normal (circles) was increased by venoclysis of hypertonic saline; in the cardiac (dots) by the intravenous injection of aminophyllin. Each datum is a single clearance period.

pressure but rather to an increased resistance to flow.

DISCUSSION

The question may be raised as to the validity of the renal clearance techniques in patients with congestive heart failure. Despite the variations in renal vasomotor activity and interstitial pressure in this condition, it has been shown (5) by renal vein catheterization that the extraction ratio of para-aminohippurate is within normal limits; hence, its clearance at low plasma concentrations may be employed as a measure of "effective" renal plasma flow. Barker and Clarke (19) have recently demonstrated that the presence of para-aminohippurate in the specimens analyzed for mannitol results in an apparent increase in the hexitol. For every milligram of para-aminohippurate in the sample analyzed, there is an ap-

parent increase of approximately 0.27 mgm. of mannitol when determined by the method (periodate-iodide-thiosulfate) employed in this study. Therefore, the glomerular filtration rates as reported in the controls are in error by approximately 2 per cent. It is somewhat smaller than this in our cardiacs, since the absolute amount of para-aminohippurate present was smaller because of the decreased blood flow. However, the relative decrease in the filtration rate in the cardiac, when compared to the normals, is unaltered by this correction.

It should be noted that the rate of sodium excretion for 24 hours must not be calculated from clearance data derived from 20- to 30-minute urine collection periods under experimental conditions. The absolute sodium intake, the glomerular filtration rate, and the effect of osmotically active solutes must be accounted for during the 24 hours. Thus, the concentration of mannitol in the urine varied from 3 to 6 per cent depending on the urine flow and, as expected from its action as an osmotic diuretic, increased the excretion of sodium from 1.2 to 2.2 times the amount in the control period without the use of mannitol. These variables help explain the persistence of edema in a patient such as P. B., who was excreting 0.11 mM of sodium per minute during the experiment, or 3.64 grams of sodium if calculated for 24 hours.

From the data in the literature, it is estimated that normally 18 per cent, or approximately $\frac{1}{5}$ of the cardiac output, flows through the kidneys. We have calculated from Merrill's (5) data that the renal fraction of the cardiac output is reduced in heart failure to 7.4 per cent or about $\frac{2}{5}$ of normal. A similar shunting mechanism occurs in other varied conditions such as orthostasis, traumatic shock (20) and chronic anemia (21), in which the common denominator is a reduced "effective" circulating blood volume, and all of which are associated with a reduced renal plasma flow and filtration rate. However, these conditions, unlike chronic congestive heart failure, are commonly associated with a decreased filtration fraction. The homeostatic mechanism of diverting blood from the kidney when the cardiac output falls is unknown. The meager data available at present seem to support the theory of a neurogenic mechanism in the early stages of cardiac failure, later sustained by a chemical mediator (22).

Since the fraction of plasma water filtered at the glomerulus is greater in congestive heart failure than has been reported in other clinical states, signifying marked efferent arteriolar constriction and increased filtration pressure, we have attempted to calculate the intraglomerular pressure in one of our severe cases of failure. We believe the results are only an approximation but they furnish a hemodynamic description of the factors involved. We have drawn liberally from Smith's (23) descriptions. We believe the patient, E. G., was able to achieve a filtration fraction (FF) of 66 per cent because of the decreased oncotic pressure due to the low plasma albumin (2.5 grams per cent). We estimate (24) the initial oncotic pressure to be 13 mm. Hg. At filtration equilibrium, the plasma proteins will have become concentrated

$$1 + \frac{FF}{1.0 - FF}$$

or 2.94 times. The equilibrium oncotic pressure will now be 13 times 2.94 or 38.2 mm. Hg. We have assumed the capsular pressure, or its equivalent, the interstitial pressure, to be about 20 mm. Smith employs 15 mm. for normal capsular pressure. We have used the larger figure because of the severe passive congestion. At equilibrium, the sum of the capsular and oncotic pressures will equal the intraglomerular hydrostatic pressure. We have, therefore, 58.2 mm. Hg for the latter or about 72 per cent of the mean (81 mm.) systemic pressure, as compared to an intraglomerular pressure of about 50 per cent of the mean systemic pressure in the normal (23). This calculation assumes a mean normal extraction ratio.

The tubular reabsorptive mechanism which maintains the plasma concentration of sodium at a fairly constant level is dependent on the glomerular filtration rate. Thus, the amount of sodium filtered (millimols per minute) is given by the plasma concentration P_{Na} (mM per cc.) multiplied by the filtration rate C_M (cc. per min.), or $P_{Na} C_M$,⁴ and is equal to the sum of the reabsorbed and excreted amounts. In equation form,

$$P_{Na} C_M = T_{Na} + U_{Na} V, \quad (I)$$

where T_{Na} is the rate of tubular reabsorption of

sodium (mM per min.); U_{Na} , the urine concentration of sodium (mM per cc.); and V , the urine flow (cc. per min.).

Dividing through by C_M , we have

$$P_{Na} = \frac{T_{Na}}{C_M} + \frac{U_{Na} V}{C_M}. \quad (II)$$

If the last term is omitted because it is minute under normal circumstances in comparison to the reabsorbed quantity of sodium, then the slope of the line obtained when T_{Na} is plotted against C_M represents P_{Na} , the plasma concentration. Under conditions of sodium administration when its excretion is increased, the second factor of Equation II is significant, under which circumstance the slope obtained represents the plasma concentration only as an approximation.

We have tried unsuccessfully on several occasions to demonstrate an overall limiting maximal capacity in the sodium transfer system by increasing the load presented to the tubules; in 1 instance the filtration rate reached a maximum of 219 cc. per minute and the plasma concentration of sodium was increased to 158 mM per liter. At all times, a constant quantity of sodium per 100 cc. of glomerular filtrate was reabsorbed. This is similar to the transfer mechanism for bicarbonate and chloride (16, 17). This dependence of the rate of sodium reabsorption upon filtration rate remains unaltered in heart failure and we must conclude that the renal tubule in congestive failure functions normally in so far as the reabsorption of sodium is concerned.

It may be questioned whether peripheral venous pressure accurately reflects the magnitude of the renal venous pressure, and whether the diminished renal blood flow may be attributable to the elevated renal venous pressure. However, if one decreases the venous pressure (peripheral, atrial and renal, presumably) with the use of mercurial diuretics, the renal plasma flow is not altered significantly. The decreased renal plasma flow is correlated with the decreased cardiac output and not with the increased atrial pressure (5). By what intermediary mechanism an altered cardiac output reflects itself in an altered renal plasma flow and filtration rate remains to be elucidated.

We believe that some of the divergent views in the literature concerning the pathogenesis of cardiac edema could be reconciled if some of the vari-

⁴ In more precise terms, the product should be written as $K \cdot P_{Na} \cdot C_M$, where K is the Donnan equilibrium constant.

ables would be controlled at the onset of the study. The patients studied by Reichsman and Grant (25) have no blood volume determinations. It is known that in any closed elastic system which is near capacity, any further tendency to increase the volume of that system first produces an increase in pressure. This may help explain the increase in venous pressure which was seen before the weight gain in their studies. Their criticism that Warren and Stead's (1) patient may have been dehydrated and that the weight gain preceding the rise in venous pressure during congestive failure represented replacement of lost water and salt, is not justified in view of the fact that there was no fluid restriction and the patient's plasma volume was 58 cc. per kgm. Landis and co-workers (26) have recently called attention to the fact that conclusions drawn from studies done under basal conditions, as in the present study, cannot be carried over in entirety to the cardiac with decreased "competence" only during activity. We agree that a rigid division between "backward failure" and "forward failure" is not consistent with all the facts. However, we are also of the opinion that salt and water retention leading to edema, secondary to a decreased glomerular filtration rate, is the major underlying factor in such diversified states as chronic congestive failure and chronic anemia. Edema would also occur in traumatic shock and orthostasis if these conditions were prolonged and normal sodium intake maintained. This sequence differs from the normal only in degree. Lyons and co-workers (27) have shown that when the normal subject ingests 40 grams of sodium chloride in 48 hours, there is a mean increase of plasma volume of 15.6 per cent and an increase of venous pressure of 31 per cent.

SUMMARY

1. The renal plasma flow and glomerular filtration rate were measured in patients with chronic congestive heart failure by the use of the paraaminohippurate and mannitol clearances. It was found that, on the average, the renal plasma flow is reduced to about $\frac{1}{3}$ and the filtration rate to $\frac{2}{3}$ of normal.

2. Under our experimental conditions, the renal tubules reabsorb a mean of 13.3 mM of sodium

from every 100 cc. of glomerular filtrate. Several patients, both cardiacs and normals, averaged somewhat higher than this mean value, while others somewhat lower. This mechanism operates in both normal man and in the patient with congestive heart failure. It offers an explanation for the relative stability of the blood sodium level.

3. In patients with congestive heart failure and in normals, physiologically induced variations in the glomerular filtration rate reveal constancy in the sodium reabsorptive mechanism.

4. The decreased excretion rate of sodium in congestive heart failure is attributable to a decreased filtration rate in the presence of normal tubular reabsorption.

We gratefully acknowledge helpful suggestions from Prof. H. W. Smith.

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EFFECT OF EXERCISE ON CARDIAC OUTPUT AND PULMONARY ARTERIAL PRESSURE IN NORMAL PERSONS AND IN PATIENTS WITH CARDIOVASCULAR DISEASE AND PULMONARY EMPHYSEMA¹

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The changes in the circulation of patients with heart disease and with emphysema have been studied many times when the patient was at bed rest. There is little information as to the changes produced when the circulatory requirements exceed the resting level. This information is probably of more importance than resting measurements as the greater part of existence is one of activity. Chronic emphysema produces changes in the heart in many patients. Congestive failure leads to water logging of the lungs. It was felt that simultaneous measurements of the cardiac output and pulmonary arterial pressure in normal persons and in those with emphysema and heart failure would aid in the understanding of the alterations of cardiac and pulmonary functions occurring in these diseases. The purpose of this paper is to present these data and to discuss their significance.

METHODS

Intracardiac catheterization was performed in the usual manner (1). The catheter was passed for a distance of 1 to 2 inches into the right or left main branch of the pulmonary artery. Pulmonary arterial pressures were recorded by a Hamilton manometer and mean pressures determined by planimetric integration, covering a period of at least 2 respiratory cycles. Atrial pressures were determined either in the same fashion, using a sufficiently sensitive Hamilton manometer, or by the use of a saline manometer. Systemic arterial pressures were measured in most cases from the brachial artery, using a Hamilton manometer. The point of zero reference was 5 cm. beneath the fourth left costochondral junction. Mixed venous blood samples were taken from the pulmonary artery in nearly all cases; otherwise, they were from the ventricle or atrium. Arterial samples were taken through an in-lying needle, usually in the brachial artery but occasionally in the radial or femoral arteries. Blood was col-

lected under oil, stored in ice, and analyzed for oxygen as rapidly as possible, using the manometric method of Van Slyke and Neill. Analyses were performed in duplicate and required to check within 0.1 vol. per cent. For the determination of oxygen consumption, 2- or 3-minute samples of expired air were collected in Douglas bags, analyzed in a Haldane apparatus, and measured in a Tissot spirometer. Exercise was carried out in the supine position by causing the patients to push with their feet against weighted pedals. The stroke distance was 12 inches. Both pedals were usually pushed together, and the total resistance could be fixed at 11, 30, or 42 lbs. The actual work rate in foot pounds per minute is of value only in indicating the general order of magnitude of the work done. The patients were not trained in this type of exercise and consequently showed obvious differences in economy of effort. The work rate was adjusted to the ability of the patient but was kept as steady as possible. Determination of the cardiac output was not begun until after at least 3 minutes of steady exercise. For the control measurements preceding exercise, no attempt was made to bring the subjects to a truly basal condition. They were, however, usually in the post-absorptive state or at most had had only a very light breakfast and had been at rest for at least 30 minutes before observations were begun. All measurements were made at 1 sitting, except in Case 24, where several days elapsed between determinations at rest and those during exercise.

RESULTS

The pertinent data on 28 subjects are presented in Table I and graphically interpreted in Figures 1 to 8.

Normal subjects

Eight persons were studied who were believed to have normal cardiovascular systems. Cases 1 to 5 had latent or asymptomatic CNS syphilis; Case 6 had a probable duodenal ulcer; and Cases 7 and 8 were convalescent, respectively, from hepatitis and pneumococcal pneumonia. The resting oxygen consumption of the group as a whole was some-

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² Holder of American College of Physicians Clinical Fellowship, 1946-1947.

CIRCULATORY RESPONSE TO EXERCISE

TABLE I

CIRCULATORY RESPONSE TO EXERCISE															
TABLE I															
No.	Case	Age	Diagnosis	Work rate	Surf. area	Atrial press.	Mean pulm. art. press.	Systemic art. press.		Blood oxygen			A-V diff.	Oxygen consump.	Cardiac index
				ft. lb./min.	sq. m.	mm. Hg	mm. Hg	mm. Hg	Sys.	Dias.	Art.	Ven.	Sat.	cc./min./M ²	L./min./M ²
									volumes per cent						
Normal															
1	W. H.	15	Latent syphilis	0	1.50	4	12	95	53	16.5	13.4	17.5	3.1	177	5.7
				760		5	14	119	67	17.3	11.5		5.8	387	6.7
2	V. W.	35	Asymptomatic CNS syphilis	0	1.85	0	8	118	70	16.0	11.6	16.9	4.4	162	3.7
				760		1	13	130	74	15.9	10.6		5.3	342	6.5
3	R. R.	20	Asymptomatic CNS syphilis	0	1.71	0	15	118	64	15.7	11.3	16.2	4.4	115	2.6
				1170			14	130	74	15.6	10.2		5.4	263	4.9
4	G. D.	30	Asymptomatic CNS syphilis	0	1.84		12	140	88	17.1	14.3	17.7	2.8	141	4.9
				920			14	110	80	17.6	11.5		6.1	314	5.2
5	R. H.	27	Asymptomatic CNS syphilis	0	1.86	0	8	116	65	19.9	16.1	20.2	3.8	177	4.7
				1430			10	150	92	19.9	15.8		4.1	398	9.7
6	H. A.	23	Duodenal ulcer	0	1.68		14	134	65	18.7	14.8	19.4	3.9	193	5.0
							12	127	71	18.7	14.5		4.2	254	6.0
7	W. W.	15	Convalescent hepatitis	0	1.58	0	11	105	74	16.1	11.8	16.8	4.3	168	3.9
				590		0	13	114	79	16.1	11.1		5.0	280	5.5
8	E. C.	21	Convalescent pneumonia	0	1.54	1	9	112	72	14.9	10.9	15.7	4.0	126	3.1
				670						14.9	8.2		6.7	285	4.2
Congestive failure															
9	D. M.	64	Hypertension, emphysema	0	1.77	6	46	154	106	16.0	9.6	20.7	6.4	183	2.8
				180			63			15.9	6.4		9.5	231	2.4
10	T. T.	40	Hypertension	0	1.64		34	206	141	16.5	12.2	17.4	4.3	157	3.6
				170			47			16.5	7.4		9.1	205	2.3
11	J. G.	63	Hypertension	0	1.54		15	146	92	17.5	11.1	19.4	6.4	167	2.6
				220			44			18.3	8.7		9.6	251	2.6
12	M. M.	47	Hypertension, pericard. effusion	0	1.50	4	42	127	108	18.0	10.8	19.1	7.2	150	2.1
				500			62			17.8	7.3		10.5	252	2.4
13	N. R.	47	Hypertension	0	2.10	8	40	207	95	13.5	9.0	16.2	4.5	164	3.7
				300		7	41			13.5	8.4		5.1	220	4.3
14	L. E.	41	Syphilitic A. I., hypertension	0	1.71		18	220	57	14.2	8.3	14.8	5.9	190	3.2
				220			29			13.4	6.2		7.2	194	2.7
15	H. S.	37	Syphilitic A. I.	0	1.70	7	40	150	80	17.0	9.1	20.7	7.9	216	2.8
				180			57			17.0	7.4		9.6	186	1.9
15A				0			27			17.9	11.7		6.2	125	2.1
				330			39			18.2	11.2		7.0	208	3.0
16	G. S.	47	Syphilitic A. I.	0	1.73	-2	25	149	55	14.1	7.4	15.1	6.7	120	2.2
				500		5	54	193	62	14.0	7.0		7.0	210	3.0

what above the normal basal level. In Cases 1 and 4 the resting A-V difference was small in proportion to the oxygen consumption. This was believed to be a result of anxiety. During exercise the oxygen consumption ranged between 250 and 400 cc. per square meter of body surface per minute. For comparison with more familiar types of activity, it may be noted that Douglas *et al.* (2) found that

TABLE I—Continued

No.	Case	Age	Diagnosis	Work rate	Surf. area	Atrial press.	Mean pulm. art. press.	Systemic art. press.		Blood oxygen			A-V diff.	Oxygen consump.	Cardiac index
								Sys.	Dias.	Art.	Ven.	Sat.			
				ft. lb./ min.	sq. m.	mm. Hg	mm. Hg	mm. Hg		volumes per cent				cc./min./M. ²	L./min./M. ²
Mitral stenosis															
17	W. N.	16	M.S., A.I.	0 1500	1.92		45(VP) 55(VP)	150 159	67 79	17.3 17.7	13.6 12.8	18.3	3.7 4.9	195 366	5.2 7.6
18	H. W.	41	M.S.	0 840	2.01	4 12	33 71	108 127	58 73	14.6 14.8	10.6 8.5	15.6	4.0 6.3	163 280	4.2 4.5
19	P. C.	36	M.S., A.F.	0 840	2.11	0 1	23 41			19.6 20.0	14.8 11.8	20.4	4.8 8.2	158 341	3.3 4.2
20	M. J.	32	M.S., M.I.	0 920	1.69	1 2	19 22	109	53	15.8 16.9	11.4 10.2	16.9	4.4 6.7	138 291	3.1 4.3
21	L. H.	21	M.S., M.I.	0	1.50		20 22	110	70	17.2 17.5	11.3 8.0	17.6	5.9 9.5	143 275	2.4 2.9
22	A. W.	36	M.S., M.I., A.F.	0 600	1.61	7 11				17.6 18.0	12.6 10.0	19.5	5.0 8.0	169 278	3.4 3.4
23	L. B.	52	Hypertension, M.S., M.I., A.F.	0	1.55	4 9	37(VP)	184	68	17.1 18.2	10.5 5.9	18.3	6.6 12.3	163 358	2.5 2.9
Pulmonary emphysema															
24	H. R.	48	Emphysema, hypertension	0	1.55	0	49(VP) 78(VP)	195	130	18.1 17.3	12.9 12.3	20.5	5.2 5.0	196 268	3.8 5.4
25	B. L.	38	Emphysema	0 1000	1.73	5 6	13 22	117 146	69 85	17.8 18.6	13.7 11.8	19.0	4.1 6.8	173 445	4.2 6.5
26	J. Y.	62	Emphysema	0 480	1.54	0 7.0	17 24	121	54	14.5 14.5	10.5 8.8	15.8	4.0 5.7	134 251	3.4 4.4
27	C. H.	47	Emphysema	0 180	1.60		24 28	114 131	74 82	16.3 16.3	10.8 9.8	18.2	5.5 6.5	184 275	3.4 4.3
28	J. A.	59	Emphysema, hypertension	0 55	1.50	1 4	18 24	170	100	15.8 15.7	12.5 11.0	18.7	3.3 4.7	149 200	4.5 4.2

the basal oxygen consumption was increased by a factor of 1.4 on standing erect; by 2.7 on walking at 2 m.p.h.; and by 3.8 on walking at 3 m.p.h. The rate of exercise in these subjects, in terms of oxygen consumption, would therefore correspond to a very slow walk on a level floor. In Figure 1 the A-V oxygen difference for each subject at rest and exercise is plotted against the oxygen consumption, the 2 points for each subject being connected by a line for purposes of identification. In order to define more completely the normal range, the extent of these values in 19 normal basal subjects observed by Stead, Warren, Merrill, and Brannon (1) is indicated on the Figure by a rectangle.

For comparison with other groups in the present study, all points, with the exception of resting values in the 2 anxious individuals, are inclosed between straight lines. The area between these lines is considered to comprise the normal range of A-V oxygen difference for the rates of oxygen consumption obtained with this type of exercise. The increased oxygen intake during exercise was achieved in part by a rise in the cardiac output and in part by an increase in the A-V difference. In 5 subjects, the rise in cardiac output was the more important factor. In the 2 anxious persons, whose A-V differences at rest were abnormally low, and in Case 8, the patient convalescent from pneu-

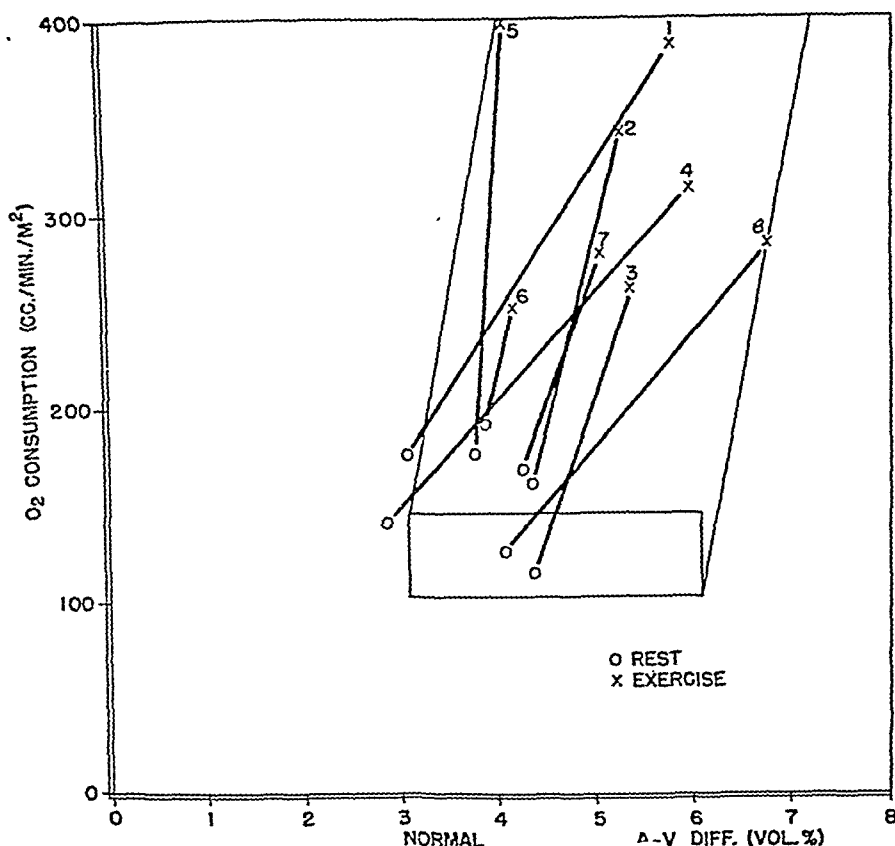


FIG. 1. EFFECT OF EXERCISE ON OXYGEN CONSUMPTION AND ARTERIOVENOUS OXYGEN DIFFERENCE IN NORMAL SUBJECTS

Numerals on this and succeeding charts correspond to subject numbers in Table I. Rectangle indicates range of values in normal basal subjects studied by Stead *et al.* (1). Region inclosed between straight lines from extremities of rectangle comprises apparent normal range of A-V difference for indicated rates of oxygen consumption induced by exercise.

monia, an increase in the A-V difference accounted for the major part of the increase in oxygen consumption on exercise. In general, during mild exercise in these subjects the A-V oxygen difference was defended against rising to large values by an increase in the cardiac output. In this group, the A-V difference did not exceed 6 vol. per cent except in Case 8, where it reached the value of 6.8 vol. per cent at a relatively low rate of oxygen consumption.

In Figure 2, the mean pulmonary arterial pressures in the normal group are plotted against cardiac index, the values for rest and exercise in each subject being connected by a straight line. Although the cardiac index extended over a range of 2.6 to 9.7 in different subjects, the mean pul-

monary pressures remained within the limits of 8 to 15 mm. Hg.

Congestive failure

Eight patients were studied who had congestive heart failure on the basis of either systemic hypertension or syphilitic aortic insufficiency. The degree of failure was, or had recently been, so severe as to compel complete bed rest. Patients Nos. 9 to 13 had hypertension, complicated in No. 9 by pulmonary emphysema and in No. 12 by pericardial effusion of undetermined cause. Patient No. 14 had hypertension and syphilitic aortic insufficiency. Patients Nos. 15 and 16 had syphilitic aortic insufficiency. Patient No. 15 was studied on 2 occasions, once while in severe failure and

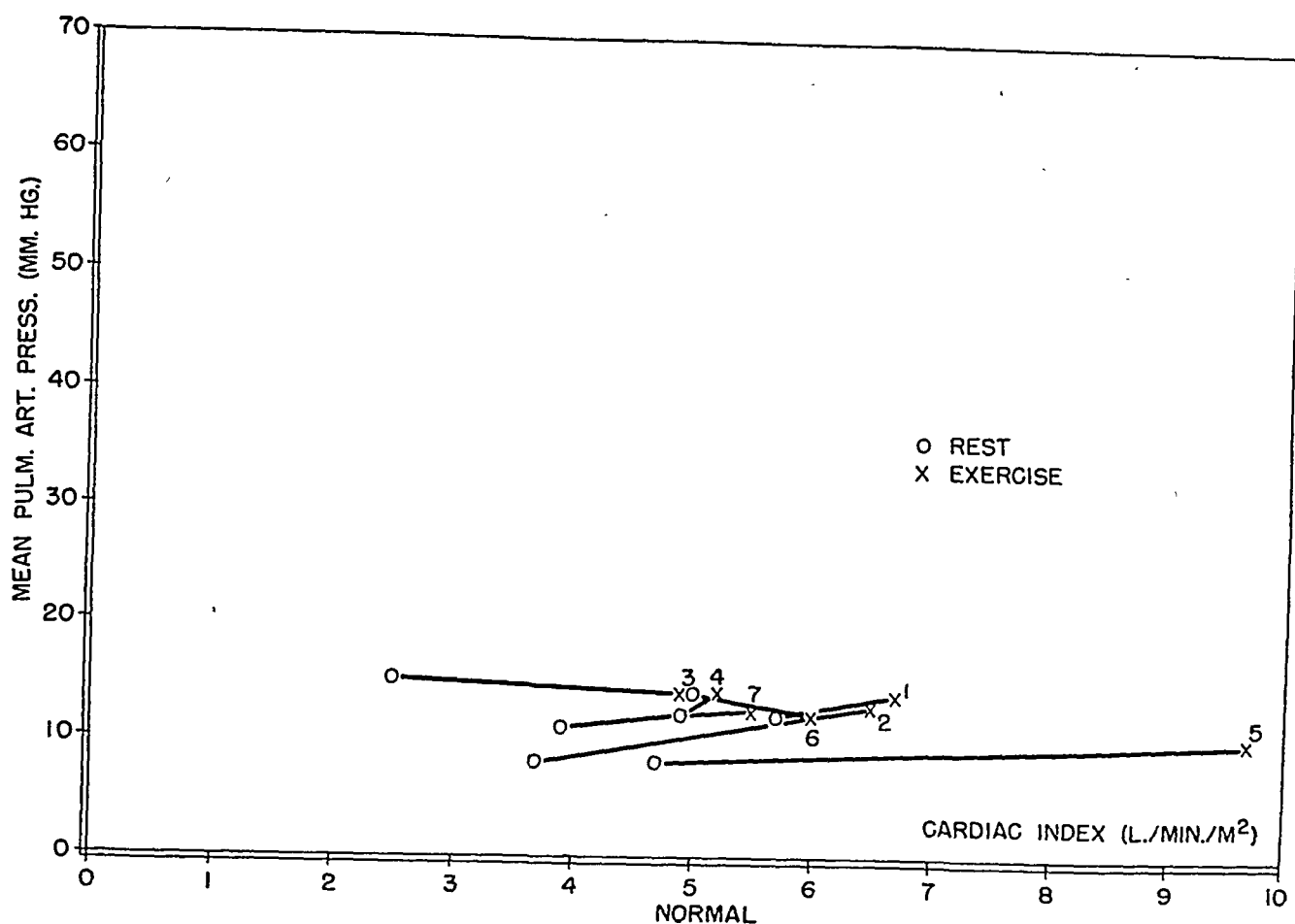


FIG. 2. EFFECT OF EXERCISE ON MEAN PULMONARY ARTERIAL PRESSURE AND CARDIAC INDEX IN NORMAL SUBJECTS

again 10 days later (15A), when he was clinically much improved. Cases 13, 15A and 16 had improved to the point of being comfortable while lying flat, while the others still had moderate orthopnea.

The ability of these patients to carry on sustained work was severely limited by weakness and dyspnea. They were encouraged to do as well as possible, and the work rate attained was regarded by most of them as a considerable effort for the 8 to 10 minutes required to complete the necessary measurements. Figure 3 indicates the relation between oxygen consumption and A-V difference in this group. The greatest rates of oxygen intake recorded were approximately 250 cc. per square meter per minute, and these were achieved only by increasing A-V differences to the region of 9.5 and 10.5 vol. per cent. With the exception of patient No. 13, all cases during exercise exhibited an A-V difference which was greater than normal for the rate of oxygen consumption, and in 3 cases this was true even at rest. Of the patients

in severe failure, 4 (Nos. 9, 10, 14 and 15) showed a slight decrease of cardiac output during exercise; 2 (Nos. 11 and 12) showed no substantial change. In 1 case there was no change in oxygen consumption during exercise, and in 1 case (No. 15) there was a slight fall. The decompensated patients, then, were severely limited in their ability to increase oxygen consumption on exercise; and whatever increase occurred was accomplished by an increase in the A-V oxygen difference. They did not respond to this type of exercise with a rise in cardiac output. The patients who had begun to improve (Nos. 13, 15A and 16), on the other hand, were able to increase their cardiac output during light exercise and thus to prevent a large rise in the A-V difference. Patient No. 13 remained within the normal range throughout.

Figure 4 demonstrates the effect of exercise on mean pulmonary arterial pressure and cardiac index. The general picture was that of an elevated pressure at rest and a very substantial further elevation on exercise. The increase of pressure

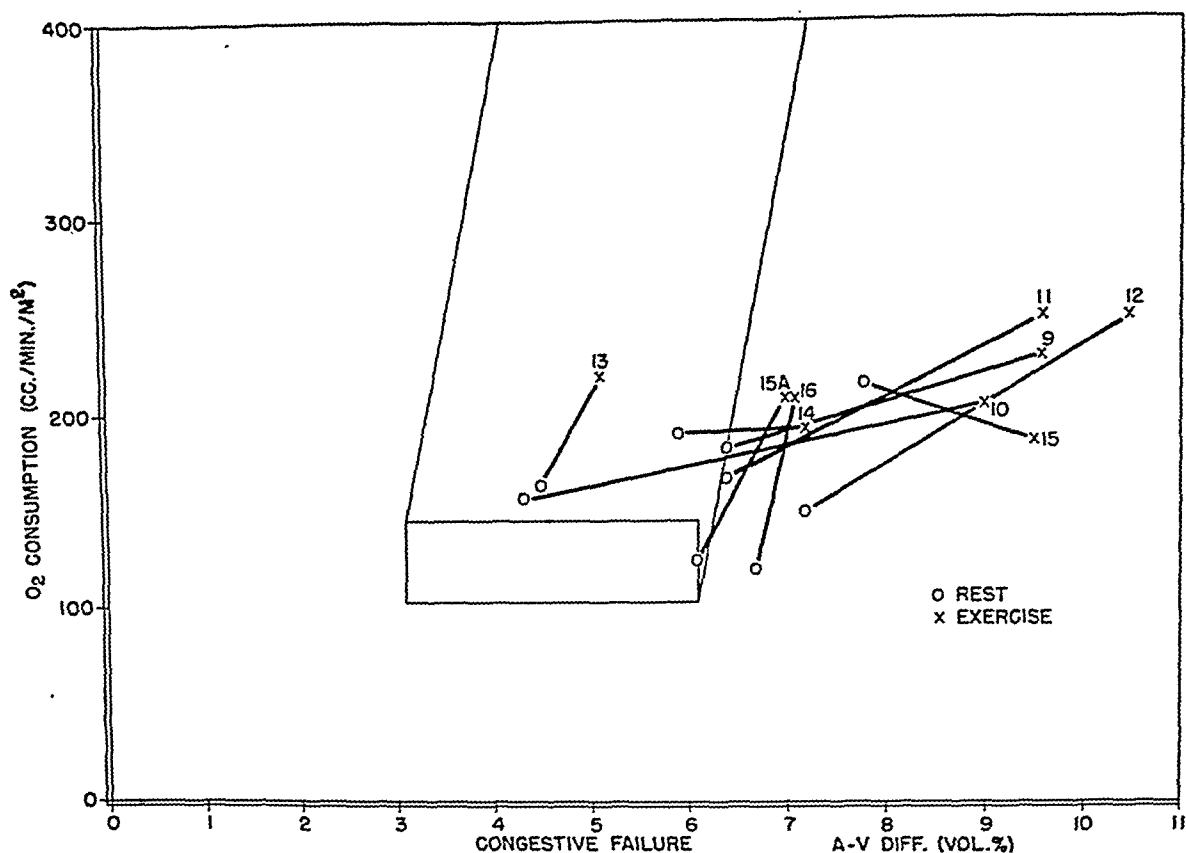


FIG. 3. EFFECT OF EXERCISE ON OXYGEN CONSUMPTION AND ARTERIOVENOUS OXYGEN DIFFERENCE IN PATIENTS WITH CONGESTIVE HEART FAILURE

Rectangle and lines indicate normal range as in Figure 1.

on exercise was independent of the direction taken by the cardiac index. In patient No. 15, the pulmonary pressure was followed at 5-minute intervals during 15 minutes of exercise and was observed to rise gradually to the recorded value.

Mitral stenosis

Seven patients were studied who had mitral stenosis. One of these (No. 17) also had aortic insufficiency and one (No. 23) had hypertension. In 2 cases (Nos. 18 and 19) only an apical diastolic murmur was present; all other cases had also a systolic murmur at the apex. Patient No. 17 had been asymptomatic. Patients Nos. 18, 19 and 20 had not had frank failure but were short of breath on exertion. Nos. 21 and 22 had had at least 1 episode of failure but were not in frank failure at the time of the study. No. 23 had had repeated failures and was recovering from his latest at the time of study.

Figure 5 relates oxygen consumption to A-V difference in this group. Only 1 patient (No. 23) had an A-V difference greater than normal for the rate of oxygen consumption at rest. On exercise 4 patients (Nos. 19, 21, 22 and 23) exceeded the normal range of A-V difference. Even in these cases, with the exception of No. 22, there was a slight rise in cardiac index on exercise, so that the increase in oxygen consumption was not entirely at the expense of the A-V difference.

Figure 6 demonstrates the relation between cardiac index and pulmonary arterial pressure in 4 of these patients. In No. 17 only the ventricular pressure is available. It was not possible to pass the catheter beyond the atrium in Nos. 22 and 23.

In all cases the right ventricular and pulmonary arterial pressures were elevated at rest and became still more elevated on exercise. Patients Nos. 18 and 19 showed very striking increases in pressure with only small increases of cardiac index,

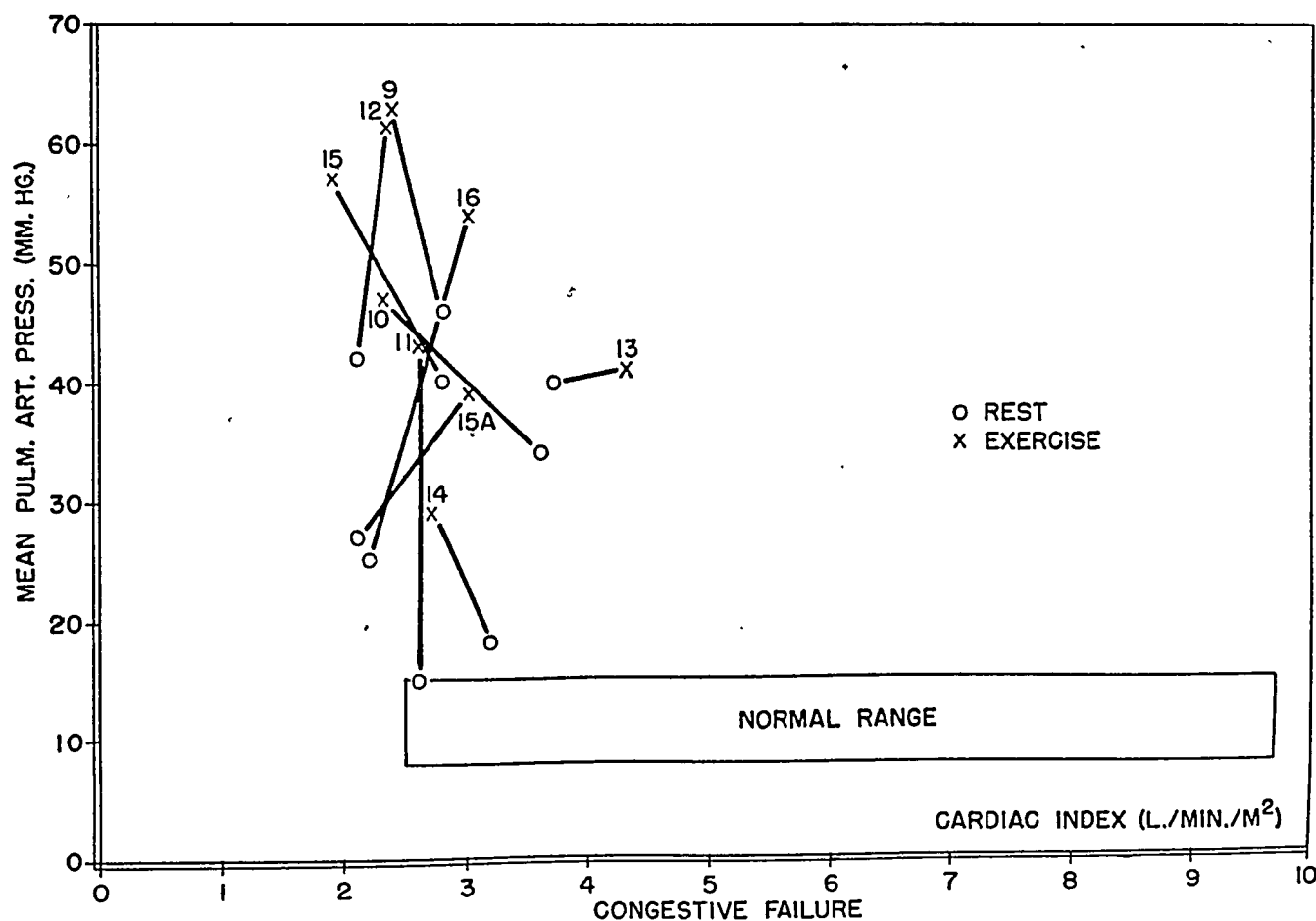


FIG. 4. EFFECT OF EXERCISE ON MEAN PULMONARY ARTERIAL PRESSURE AND CARDIAC INDEX IN PATIENTS WITH CONGESTIVE HEART FAILURE

Rectangle incloses range covered by normal subjects during rest and exercise.

thus resembling the behavior of the group with left ventricular failure.

Pulmonary emphysema

Five patients (Cases 24 to 28) were studied who had well advanced pulmonary emphysema. The diagnosis was made by physical examination and X-ray findings. In addition, Cases 24 and 28 had systemic hypertension, but no evidence of cardiac enlargement. Figure 7 indicates the relationship between oxygen consumption and A-V difference for the rates of oxygen consumption encountered.

The relationship between mean pulmonary pressure and cardiac index is summarized in Figure 8. With a single exception, the resting pressures exceeded the normal, and on exercise there was a substantial further increase in pressure, well exceeding the normal range in each case. In all cases but No. 28, this increase in pressure was associated with an increase in the rate of blood flow through the lungs. The behavior of No. 28 resembles that

of a person with congestive heart failure, but the changes in oxygen consumption and A-V difference during exercise were too small to allow definite conclusions.

DISCUSSION

Response of cardiac output to exercise

With exercise there is increased demand of the tissues for oxygen. This demand is met by increasing the amount of oxygen uptake in the lungs either through an increase in cardiac output or through an increase in arteriovenous oxygen difference so that more oxygen is removed by each unit of blood passing through the lungs. During light exercise, normal subjects may use both mechanisms to some extent but the rise in cardiac output predominates and the change in A-V oxygen difference is minimized. In persons with an inadequate heart the expected rise in cardiac output cannot occur and the A-V difference is greatly increased.

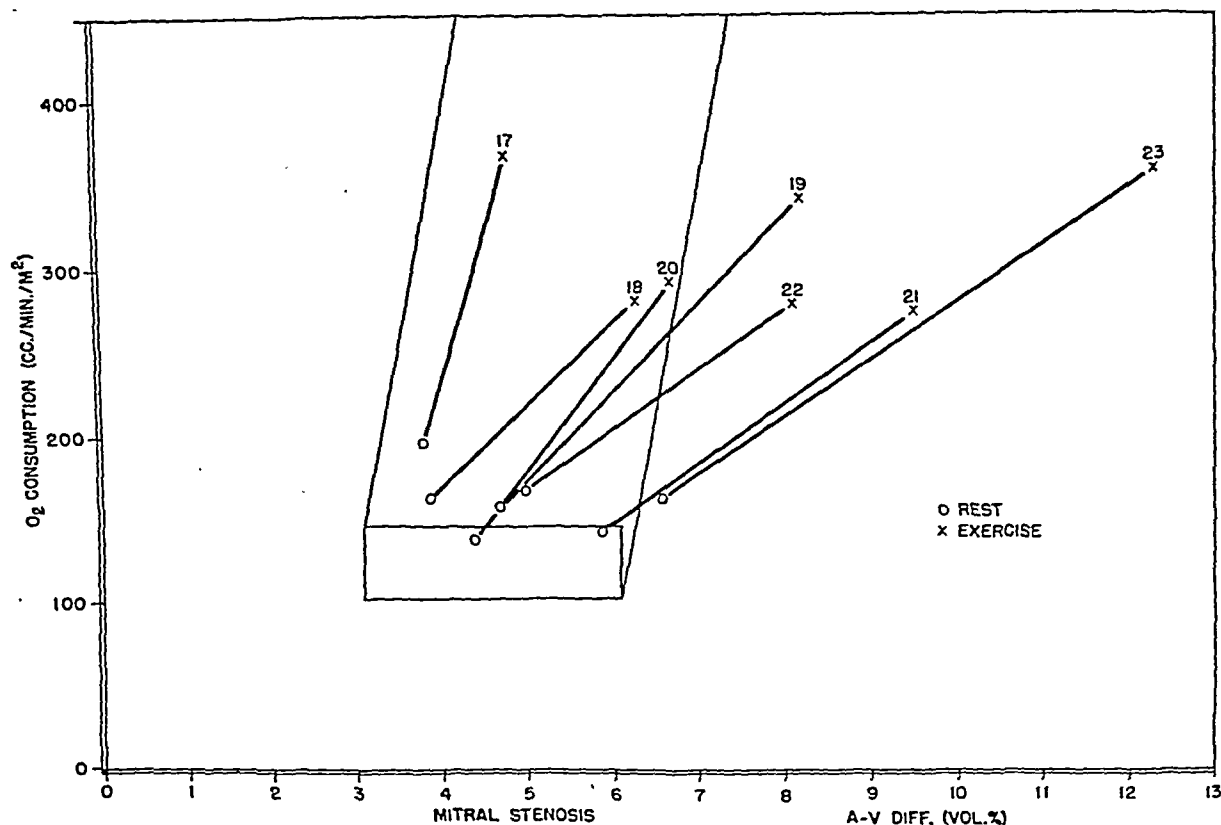


FIG. 5. EFFECT OF EXERCISE ON OXYGEN CONSUMPTION AND ARTERIOVENOUS OXYGEN DIFFERENCE IN PATIENTS WITH MITRAL STENOSIS

Rectangle and lines indicate normal range, as in Figure 1.

All investigators have found that the resting cardiac output is usually decreased in patients with intractable congestive failure. There is some overlap in the values for normal resting subjects and persons with congestive heart failure. This would be expected because the cardiac output is measured under conditions in which the requirements for blood are at a minimum. It would be expected that the difference in cardiac output between normal persons and those with heart failure would be more pronounced if the values for cardiac output in both groups were compared during light exercise. The values for resting cardiac output in normal subjects and in the persons with chronic congestive heart failure reported here do overlap, but the difference between the 2 groups is increased by exercise.

In normal persons during light exercise, there is considerable variation in the cardiac output from one subject to the next. In order to compare the normal response to exercise with the

response of the group in congestive failure, it is necessary to choose some criterion to determine whether the cardiac output is adequate for the blood requirements of the body. The level of the arteriovenous oxygen difference is a convenient criterion. If this quantity can be maintained within normal limits while the rate of oxygen consumption is substantially increased, it follows that the response of the cardiac output has been sufficient to maintain a normally high oxygen tension in the tissues. Based on the present results, an upper limit of normal has been chosen for the A-V difference during light exercise. The use of this criterion must be somewhat qualified. It is valid, of course, only if the blood oxygen capacity is within the normal range. As the severity of exercise increases, the A-V oxygen difference may be expected to increase in a normal person. Because of this, the level of A-V difference chosen as a criterion must be adjusted according to the severity of the exercise. Finally,

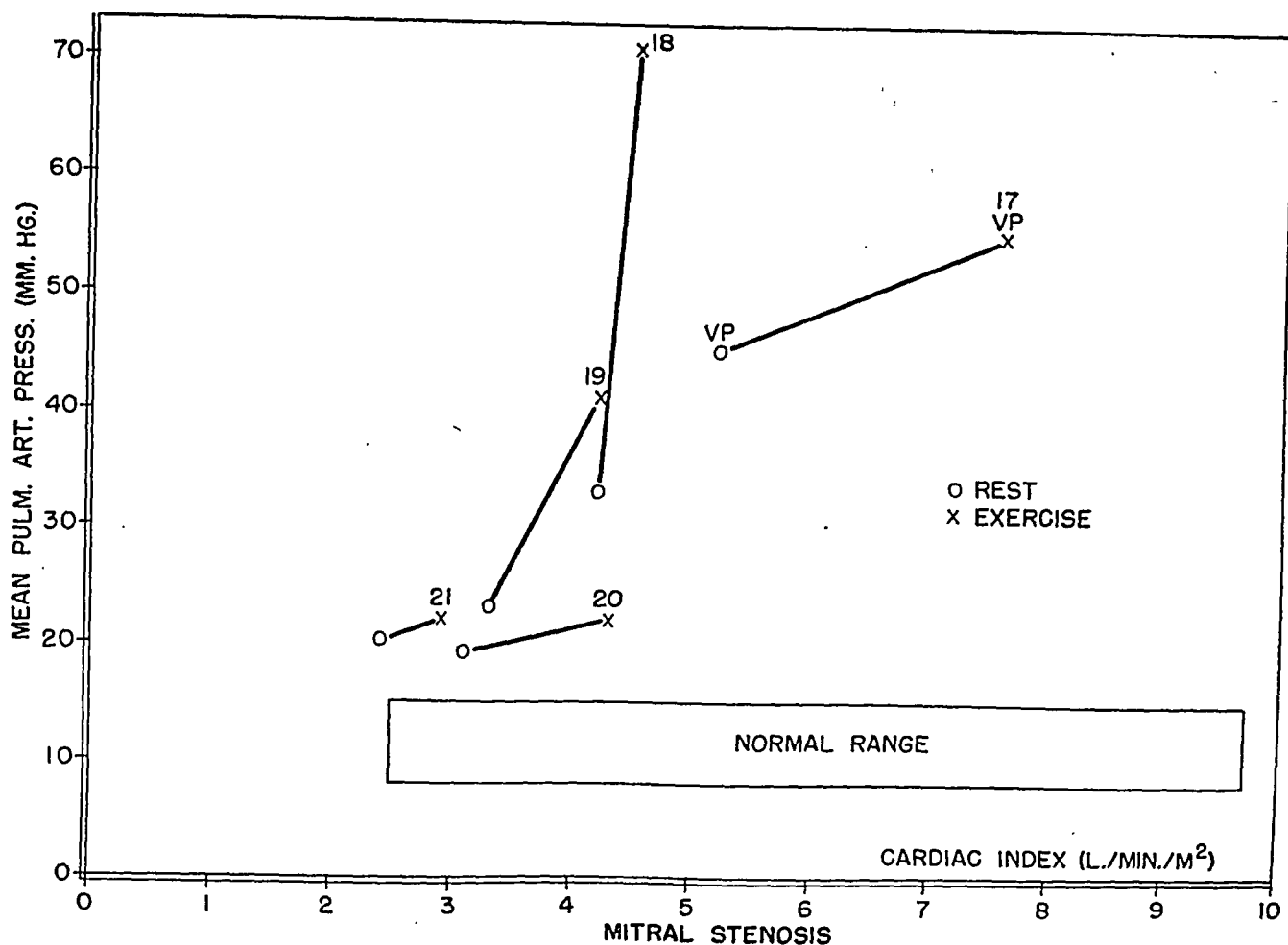


FIG. 6. EFFECT OF EXERCISE ON MEAN PULMONARY ARTERIAL PRESSURE AND CARDIAC INDEX IN PATIENTS WITH MITRAL STENOSIS

Rectangle incloses range covered by normal subjects during rest and exercise. "VP" indicates right ventricular systolic pressure.

the level of A-V difference which is used as a criterion for adequacy of the cardiac output cannot be applied to persons who have, in addition to congestive failure, disorders such as thyrotoxicosis and anemia which in themselves affect the A-V difference.

In the present group of normal persons, when the rate of oxygen consumption was increased by exercise to 250 to 400 cc. per square meter per minute, the largest A-V oxygen difference was 6.8 volumes per cent. This occurred at a rate of oxygen consumption of 285 cc. per sq. m. per min. On Figure 1 a line is drawn between this point and that point of the normal basal region which represents the largest A-V difference (6.1 vol. per cent) and the lowest rate of oxygen consumption (103 cc. per sq. m. per min.). This line is selected as a boundary which separates the region where cardiac output is probably adequate for the

demands made by muscular exercise from the region where output is probably inadequate.

By this criterion the group of patients with congestive failure (Figure 3), with a single exception, had an inadequate cardiac response to exercise, and in 6 cases (Nos. 9, 10, 11, 12, 14 and 15) the response was grossly inadequate. In 3 cases, as judged by the A-V difference, the output was not normally commensurate with the metabolic requirement even at rest. This inability of the heart to respond normally to an increased metabolic requirement is made more striking by the low value of the requirement which was imposed. The largest rate of oxygen consumption attained in this group was 250 cc. per sq. m. per min. The 1 patient (No. 13) who remained within the normal range achieved an oxygen consumption of only 220 cc. per sq. m. per min.

During exercise in the group with congestive

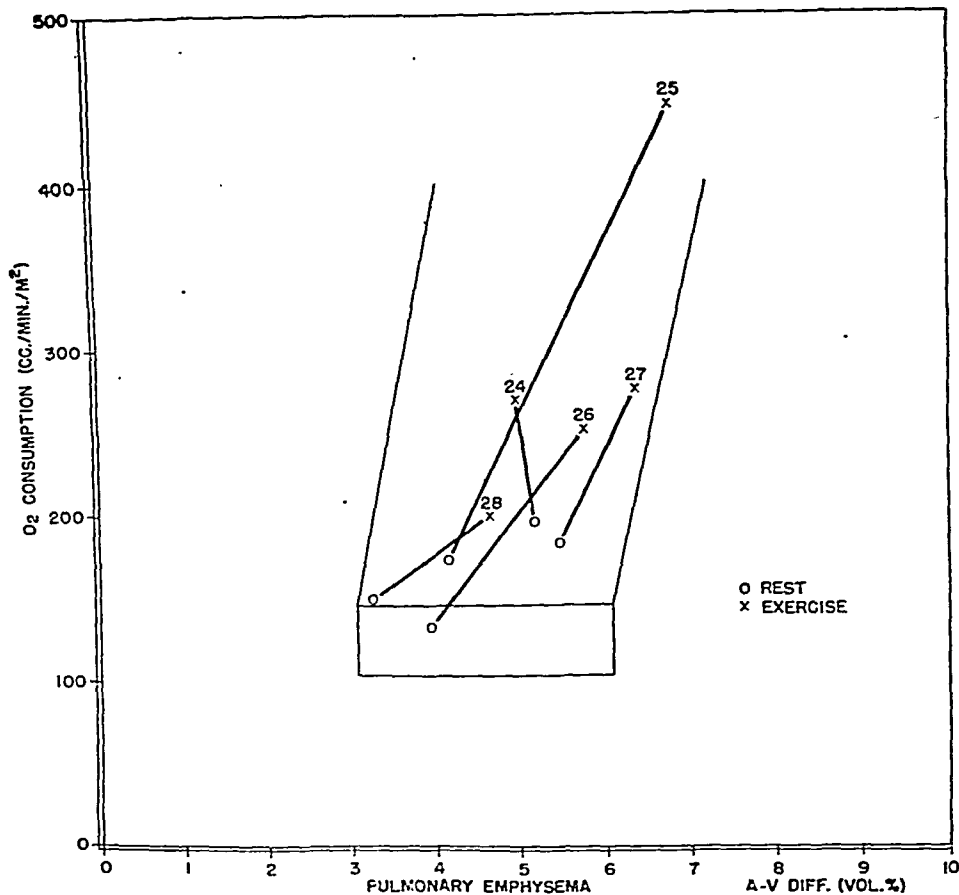


FIG. 7. EFFECT OF EXERCISE ON OXYGEN CONSUMPTION AND ARTERIOVENOUS OXYGEN DIFFERENCE IN PATIENTS WITH PULMONARY EMPHYSEMA

Rectangle and lines indicate normal range, as in Figure 1.

failure the cardiac output rose slightly above the resting level in 4 cases, remained unchanged in 1, and fell slightly in 4 cases. In all these cases, the venous blood samples used in determination of the output were taken from the pulmonary artery, where mixing should have been complete. At first sight it seems paradoxical that there should be a fall in cardiac output during the stimulus of exercise. The determination of output, however, was begun only after exercise had been continued for several minutes. It appears probable that the values obtained for output during exercise in the patients with frank failure do not represent a state of equilibrium. It is suggested that the output during steady exercise may rise at first, but then decline even below the resting value as the myocardial reserve is exhausted by the initial sprint.

The significance of the low resting cardiac out-

put which occurs in most types of congestive failure is greatly increased by the finding that this is apparently the largest output which the heart can long maintain. Under these circumstances, requirements for extra blood flow to a particular system, such as the muscular structure, can only be met by diverting blood flow from regions in which essential metabolic functions are being conducted. This diversion must result in postponement of many such functions without the possibility of a compensatory increase in blood flow when the extra requirement has passed. It was found by Meakins and Long (3) that following a standard work period the recovery time of cardiac patients, in terms of increased oxygen consumption was greater than that of normal subjects. From this and from the finding of a high blood lactic acid level in congestive failure, they concluded that a decompensated cardiac patient may be in a state

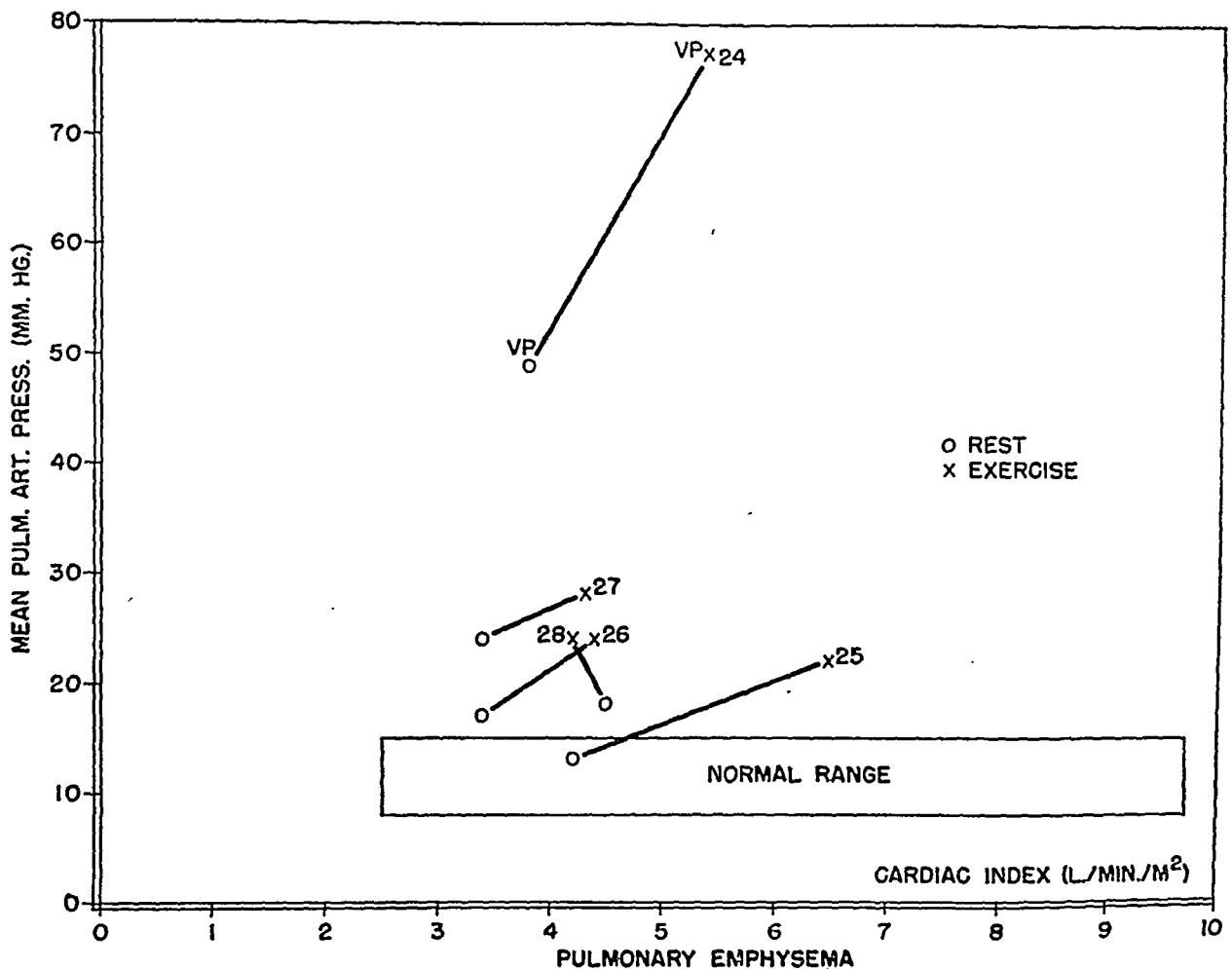


FIG. 8. EFFECT OF EXERCISE ON MEAN PULMONARY ARTERIAL PRESSURE AND CARDIAC INDEX IN PATIENTS WITH PULMONARY EMPHYSEMA

Rectangle incloses range covered by normal subjects during rest and exercise. "VP" indicates right ventricular systolic pressure.

of chronic oxygen debt. The present results confirm this conclusion. The presence of a metabolic need to which the heart is not adequately responding is indicated by the frequent finding of an abnormally large A-V difference at rest in patients with the ordinary types of congestive heart failure.

In summary, the resting cardiac output in frank congestive failure is the greatest that can be consistently maintained but still may not be great enough to supply the tissues with blood at a rate normally commensurate with their metabolic needs. Any important requirement for additional blood flow cannot be met by increasing the cardiac output but only by diverting blood from one system to another.

Of the patients with mitral stenosis (Figure 5), the A-V difference exceeded the normal range

during exercise in 4 instances, indicating a sub-normal cardiac response for the amount of work done. Three of these patients (Nos. 21, 22 and 23) had had at least 1 episode of frank congestive failure. The remaining patient (No. 19) had dyspnea on exertion. Of the 3 patients who remained within the normal range, none had ever been frankly decompensated, although No. 18 and No. 20 had dyspnea on exertion. No. 17 had been asymptomatic.

In the group of patients with pulmonary emphysema (Figure 7), the A-V difference remained within the normal range for the rates of oxygen consumption achieved, indicating that the cardiac response was adequate for the requirement. In patients with pulmonary emphysema the problem frequently arises as to whether there is co-existent heart failure. In such cases the pres-

ent method should distinguish those whose cardiac function is adequate within the limits of the work imposed from those whose function is inadequate.

The pulmonary arterial pressure

The mean pressure in the pulmonary artery depends upon several factors. It is the sum of the left atrial pressure and the pressure gradient between artery and atrium. The gradient, by Poiseuille's law, is directly proportional to the cardiac output and inversely proportional to the "peripheral resistance," a factor which depends primarily upon the number and caliber of small vessels in the pulmonary bed. Finally, in measurement, the mean pulmonary arterial pressure is affected by the mean intrathoracic pressure. The cardiac output was determined in the present study but the relative effects on pulmonary arterial pressure of the left atrial pressure, the condition of the vascular bed (peripheral resistance), and the intrathoracic pressure must be inferred from other evidence.

In normal man, when the cardiac output is increased by exercise up to twice the resting level there is no change, or a small increase in mean pulmonary arterial pressure. As a rule, however, the proportional increase of output is much greater than that of pressure. Accordingly, there must be a compensatory decrease in resistance of the vascular bed, the left atrial pressure, or the mean intrathoracic pressure. Because the absolute value of the pulmonary pressure is quite low, it is difficult to determine the relative importance of these factors. Hamilton (4) has found that the pulmonary bed of the dog can accommodate a large increase in blood flow without a proportional rise in pressure gradient, indicating a decrease in the resistance. In man, Cournand (5) has found that the right ventricular pressure is not significantly increased after pneumonectomy. These observations suggest that the ability of normal man to increase the rate of pulmonary blood flow without a corresponding rise in arterial pressure results from the possibility of opening new vascular channels or of widening those already open.

In the patients with congestive failure, the pulmonary arterial pressure was, on the whole, markedly elevated at rest and showed a striking further increase during exercise. The cardiac output, on

the other hand, was low at rest and was not much altered during exercise. In the presence of a low, relatively fixed cardiac output, the high pulmonary pressure and its further elevation on exercise must be referred to a large increase of left atrial pressure, pulmonary resistance, or both. In general, the pressure elevations are of such magnitude as to exclude increases of mean intrathoracic pressure as a significant factor. Certain observations suggest that elevation of the left atrial pressure, or left ventricular diastolic pressure, is responsible in part for the pulmonary hypertension which occurs in congestive heart failure. In persons who die with congestive failure, the pulmonary capillaries are dilated with blood and many more are visible than in the normal lung. This finding demonstrates a low resistance of the capillary bed itself and at the same time suggests a high intracapillary pressure. The experiments of Hamilton (4) have demonstrated that in the dog a very large increase in left atrial pressure results in an equal rise of pulmonary arterial pressure. Some evidence that the high pulmonary arterial pressures of congestive failure are partly transmitted to the capillaries is provided by a patient with systemic hypertension and congestive failure whose case has not been included with the others because the data were incomplete. This patient developed a prolonged attack of acute dyspnea after 6 minutes of light exercise, requiring termination of the experiment. The mean pulmonary arterial pressure at rest had been 42 mm. Hg. The pressure during the attack was not measured, but 5 to 10 cc. of blood was driven through the No. 8 catheter into the attached tubing against a gravity head of approximately 90 mm. Hg, resulting in clotting within the catheter. The passage of this quantity of blood through a No. 8 catheter requires a prolonged elevation of pressure. This patient had had spontaneous attacks of paroxysmal dyspnea on the ward. The association of this symptom with acute pulmonary edema is well established, and its occurrence together with a great rise in pulmonary arterial pressure suggests that at least a portion of this pressure was transmitted to the capillaries, resulting in a rapid filtration of fluid through their walls. It is not yet possible to quantitate the role played by constriction of the pulmonary arterioles or the influence on pulmonary pressure of

stiffening of the lung structure resulting from pulmonary edema.

In the group of patients with mitral stenosis it was anticipated that an increase in the rate of blood flow through the lungs would be attended by a greater than normal increase in pulmonary pressure because of obstruction to outflow from the left atrium. In the 4 cases where it was possible to catheterize the pulmonary artery, the pressures were, in fact, elevated at rest and rose still further when the output was increased by exercise. In 2 of these cases (Nos. 20 and 21) the resting pressures were not greatly elevated, and the increase in blood flow on exercise was proportionately greater than the increase in pressure. The remaining 2 patients (Nos. 18 and 19) demonstrated increases in pulmonary pressure during exercise which were, proportionately, 100 per cent and 50 per cent greater than the respective increases in cardiac output. These pressure changes were of large absolute magnitude. The finding of a pressure rise so disproportionate to the increase in blood flow indicates that some factor in addition to a small, fixed mitral orifice or pulmonary arteriosclerosis must have been operating to produce the pressure increment on exercise. If the resistance of the pulmonary bed and the left ventricular diastolic pressure had remained unchanged, then the rate of flow through the mitral orifice should have been roughly proportional to the mean pulmonary arterial pressure. The large rise in pulmonary pressure without a corresponding increase in cardiac output resembles the behavior of the group with left ventricular failure. The mechanism by which left ventricular failure might occur in mitral stenosis is not immediately apparent. There was no evident cause for cardiac disease other than mitral deformity in these 2 patients, and in neither of them could an apical systolic murmur be heard.

In pulmonary emphysema the existence of pulmonary hypertension has been postulated from the histological picture on the basis that the vascular bed becomes greatly narrowed as the result of destruction of small vascular channels. This narrow bed would then require a higher than normal pressure gradient to maintain a normal rate of flow. Further increments in flow would require greater than normal increments in pressure since the capacity for widening the vascular bed has

been largely exhausted. The present results appear to be in accord with this concept. The mean arterial or ventricular systolic pressures were higher than normal at rest except in 1 case. On exercise there was a further rise in pressure which in 4 cases was sharper than normal in proportion to the rise in cardiac output. In the remaining case (No. 28) the pressure rose, while the cardiac output showed a very slight fall. This sequence of events resembles that in congestive heart failure, where the increase in pulmonary arterial pressure on exercise is believed to result largely from a pressure rise in the left atrium. Although patient No. 28 had no signs of congestive failure, she was an elderly woman with a long history of systemic hypertension. It is considered likely that the atypical behavior in this case was the result of an increase in left atrial pressure on exercise. In general, it appears unlikely that large rises in left atrial pressure or in mean intrathoracic pressure could account for the high resting pulmonary arterial pressures in emphysema or for the sharp increase in pulmonary pressure on exercise. The rarity of pulmonary edema during life or of pulmonary vascular congestion at autopsy in these patients argues against the frequent occurrence of a high left atrial pressure. Large changes in the mean intrathoracic pressure should be reflected to some extent in the right atrial pressure. The right atrial pressure (Table I) was not elevated in most of these patients at rest. During exercise it rose, but the rise, except for 1 case, was substantially less than that of the mean pulmonary arterial pressure. For example, in a separate experiment on patient No. 24, it was found that the average right ventricular diastolic pressure increased by 4 mm. Hg while the ventricular systolic pressure increased by 12 mm. Hg on passing from rest to exercise. In summary, the available evidence indicates that pulmonary hypertension in emphysema results from narrowing of the vascular bed so that a high pressure gradient is required to force blood through the lung at a normal rate. It should be noted that a normal resting pulmonary arterial pressure in a patient with emphysema does not establish normal function of the pulmonary vascular bed. As illustrated by patient No. 25, pulmonary hypertension may appear only when the rate of blood flow is increased.

SUMMARY AND CONCLUSIONS

1. Determinations of cardiac output by the Fick principle and of mean pulmonary arterial pressure were made at rest and during light exercise in 8 persons with a normal cardiovascular system, 8 cases of congestive heart failure due to systemic hypertension or syphilitic aortic regurgitation, 7 cases of mitral stenosis, and 5 cases of pulmonary emphysema.

2. In normal persons during exercise there is an increase in both cardiac output and arteriovenous oxygen difference, but the increase in cardiac output predominates.

3. In persons with congestive heart failure there is little or no increase in the cardiac output during exercise, but there is a large increase in arteriovenous oxygen difference.

4. In frank chronic congestive heart failure the resting cardiac output is the greatest that can be consistently maintained, but even at rest this output may not be great enough to supply the tissues with blood at a rate normally commensurate with their metabolic needs.

5. The cardiac response to exercise may be considered adequate if the arteriovenous oxygen difference can be maintained within normal limits for the rate of oxygen consumption achieved. When the cardiac response is inadequate, the A-V difference exceeds the normal limits.

6. The normal pulmonary vascular bed can accommodate a large increase in the rate of blood flow with little or no increase in mean pulmonary arterial pressure.

7. In persons with left ventricular failure, the mean pulmonary arterial pressure is elevated at rest and shows a large further elevation during exercise, with little or no increase in the cardiac output. It is believed that this pressure elevation

results in large part from an increase in left ventricular diastolic pressure.

8. In well-marked mitral stenosis the pulmonary arterial pressure is elevated at rest and is further increased during exercise. In certain patients the increase of pulmonary pressure during exercise is greater than can be accounted for on the basis of a fixed obstruction at the mitral orifice.

9. In advanced pulmonary emphysema the pulmonary arterial pressure is usually elevated at rest and becomes still further elevated during exercise. In patients with emphysema who do not have congestive heart failure, pulmonary hypertension is believed to result from destruction of small vessels in the lung with consequent narrowing of the pulmonary vascular bed.

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A STUDY OF THE HUMAN MYOGRAM. A STUDY OF NORMALS, AND OF PATIENTS WITH ADDISON'S DISEASE, THYROTOXICOSIS AND PROGRESSIVE MUSCULAR ATROPHY¹

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A simple method of myography is often needed in the clinic to confirm one's impression of impaired or normal muscle function. The method should not depend upon the patient's voluntary performance, and should not be so painful that repeated testing is objected to by the patient. In the present study a method of myography similar to that used in animal experiments has been employed. Without anesthesia the ulnar nerve was electrically stimulated at the elbow by means of skin electrodes. The maximum contractions of the muscles in the forearm and hand innervated by the ulnar nerve (flexor carpi ulnaris; ulnar head of flexor digitorum profundus; and intrinsic muscles of the hand) were measured mechanically by an isotonic myograph and recorded on a slowly moving paper by an ink writer.

Because of the pain and consequent variability of the muscular contractions which attended high frequency stimulation of the ulnar nerve, the present method utilized stimulation frequencies varying from once every 10 seconds to 8 per second. Even at these slower frequencies pain was frequently sufficiently severe to render the muscle curves of little use. In approximately half of the subjects, however, reliable myographic tracings were obtainable and, in each of these tests, the curves for the same individual were practically identical on repeated tests.

METHOD

The subject was seated comfortably and the right or left forearm was fitted firmly without constriction into a rigid supporting frame (Figure 1). The elbow was flexed at a right angle and the forearm was semi-pronated. To allow freedom of movement, the wrist

extended beyond the end of the frame. A leather strap around the hand was connected to the myograph by a cord over a pulley. The tension of the myograph was varied by elastic bands of different stiffness which required weights of approximately 300 to 1000 grams to produce a deflection of the myograph equal to a single muscular twitch. The stimulating electrode was a wedge-shaped piece of solder about 2 cm. long. It was grooved for electrode paste. The electrode was securely taped over the ulnar nerve where it lies between the medial condyle of the humerus and the olecranon. The electrode was usually placed in the inferior part of the fossa as distant as possible from the sensory nerves which emerge superiorly. The indifferent electrode was a piece of copper screening 7.5 by 15 cm. covered by cheese cloth and soaked in salt solution. It was placed on the opposite arm. The stimuli consisted of monophasic square wave discharges of one millisecond duration produced by a Grass stimulator.³ Stimuli of longer duration were too painful while stimuli of shorter duration did not give consistent optimal responses. The strength of stimulation varied with the individual and ranged from 20 to 65 volts. It was relatively constant for the same person on repeated tests.

The following routine was finally adopted for test purposes. At the beginning of each experiment, stimuli were delivered every 10 seconds, until the voltage giving the maximum mechanical response was found. Suboptimal mechanical responses were produced by excessively strong as well as by submaximal stimuli. In the former instance it was due to spread of the current to antagonistic muscles and to apprehensive fixation of the wrist and forearm by the subject incident to the pain of strong electrical stimulation. After selection of the optimal voltage the ulnar nerve was stimulated once every 2 seconds for 1½ minutes. This test period was followed by a rest period during which stimuli were delivered every 10 seconds. This continued until the potentiation resulting from the test run had disappeared and the height of contractions had returned to their former level. The rate of stimulation was increased to 1, 2, 4, 8, and occasionally 16 per second during succeeding test runs, usually of 1½ minutes' duration. Between these periods of rapid stimulation shocks were given every 10 seconds until the height of contraction fell to a constant level.

¹ This study was aided in part by a grant from the Committee on Research in Endocrinology, National Research Council.

² Commonwealth Fund Fellow, 1945-1947.

³ This instrument was supplied to us by the Grass Instrument Co., Quincy, Mass.

A STUDY OF THE HUMAN MYOGRAM

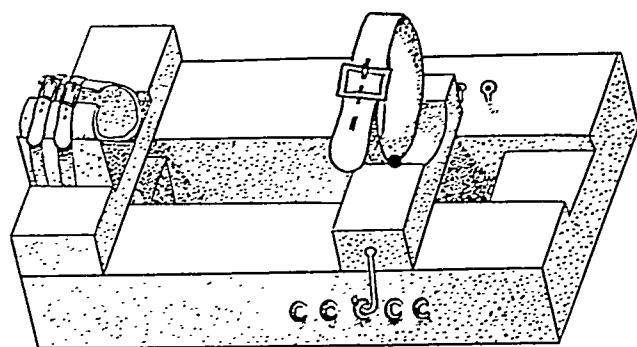


FIG. 1. A DRAWING OF THE ARM HOLDER

The single wide strap at the right fits loosely around the forearm just below the elbow. The 2 narrow straps to the left secure the wrist, the head of the radius being held firmly against the upper end of the angulated block of wood shown in cross-section. The wrist is wrapped by a layer of felt about $\frac{1}{8}$ inch thick to protect it from the wooden block. Three angulated wood blocks were available, one 60°, one 45°, and the other 30° from the horizontal. The one shown in the insert (approximately 60°) usually gave the best responses. The leather loop to the left of the wrist holder fits around the hand which is held slightly extended. A cord extends from this leather loop over a pulley to the myograph which records horizontally, by an ink writer 5 inches long, on slowly moving paper.

RESULTS

1. Observations on normal subjects.

The strength of a single muscular contraction seemed to correlate with the general muscular strength of the subjects being tested. Figures 2A, B, and C show a typical series of tests in normal subjects (A and C females; B male). The first few single muscle contractions were 10 seconds apart. The first test was made at a stimulation rate of 0.5 per second. Throughout this run there was a gradual increase in the strength of each succeeding contraction, the phenomenon of potentiation. The first, and maximum, contraction during the recovery period which followed this test was equal to the maximum contraction during the test. Following this there was a gradual decrease in the contraction height (loss of potentiation) to the strength (height) which preceded the test.

With increase in the rate of stimulation to 1, 2, and 4 per second the build-up in contraction height occurred at a more rapid rate, and the maximum potentiation was reached long before the end of the 1½-minute test period. With 1 and 2 per second, and occasionally at 4 per second frequencies, the maximum potentiation was maintained to the end of the test run. In most 4 per second tests the height of contraction fell off near the end of the test. At 8 per second there was usually an early partial fusion of tetanus which occasionally became completely fused. At 16 per second, fusion of tetanus occurred in all of our tests, pain usually limiting the application of stimulus at this rate for periods longer than 3 seconds (Figure 2F).

The first single contraction after the 1 and 2 per second tests usually equalled the height of the maximum contraction attained during the preceding

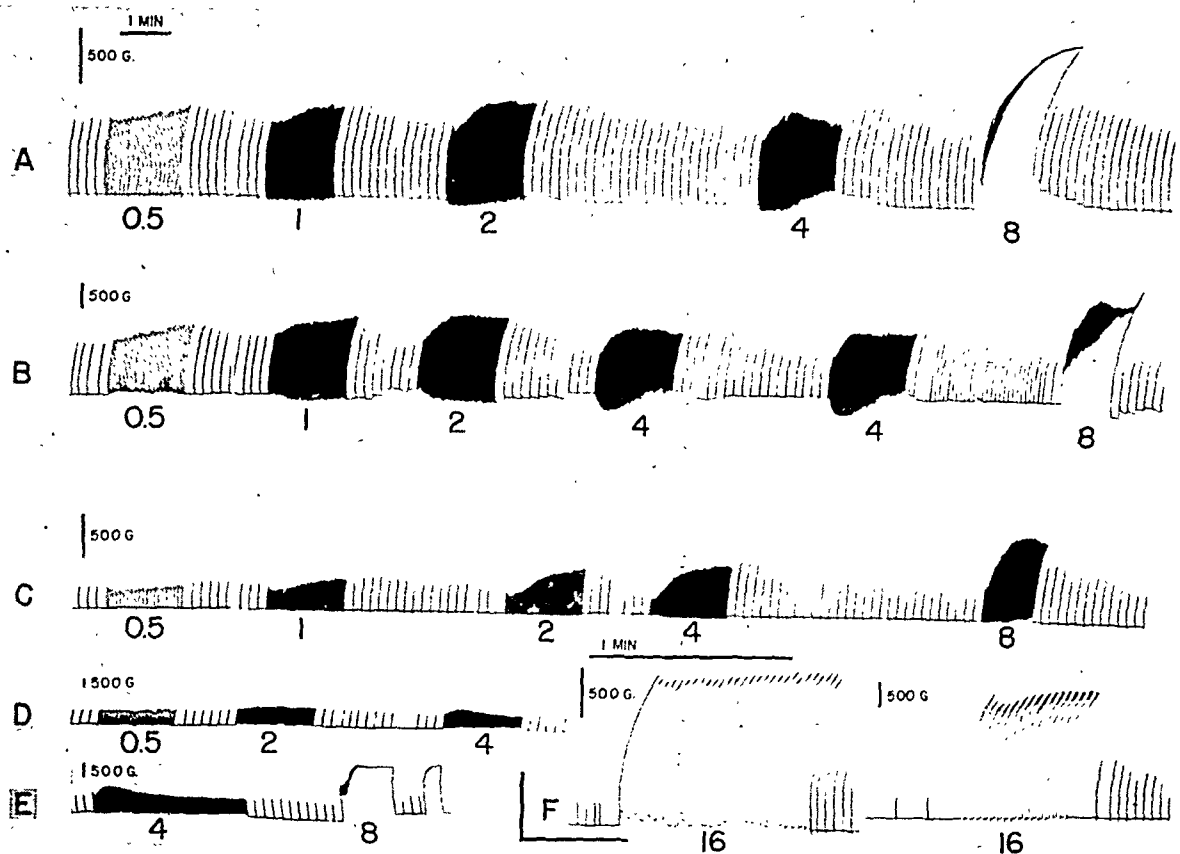


FIG. 2. MYOGRAMS OF NORMAL SUBJECTS

The tension of the myograph is shown in the left upper corner of each record. The number below each record indicates the frequency of stimulation per second. A. Record of a normal woman. B. Record of a normal man. Note the similarity of the two 4 per second tests. C. Record of a normal woman. D. Same subject as in B. Torsion wire myograph with nearly isometric muscle contractions. E. Same subject as B. Isotonic myograph; increased tension. F. Two records at 16 per second frequency. Stimulation for 3 seconds alternating with rest for 3 seconds.

ing test run. At 4 per second this was occasionally true. In most subjects, however, the first one (or few) single contraction after a 4 per second test run was of lower height than the maximum contractions during the preceding test run. The contraction height then usually increased to this maximum before recovery from potentiation began. During the recovery period, the loss of potentiation occurred rapidly at first and then more slowly as the resting contraction height was approached. Recovery from potentiation was slower in males than females. In all subjects the time of recovery, *i.e.*, the restoration of the height of contraction to the baseline level, was inversely proportional to the stimulation speeds or to the degree of potentiation. The potentiation produced by stimulation at a rate of 0.5 per second often was not completely lost during the following recovery

period when the contractions occurred every 10 seconds.

In most physically well-trained and non-fatigued subjects a steady increase in the height of contractions was observed during the slow tests. In many subjects, however, early in the 2, 4, and 8 per second tests the height of contractions fell off from the beginning for a short space and was then followed by a secondary rise in the contraction height (Figure 2C). This early dip in the contraction height was most marked at the more rapid frequencies. It became more prominent after repeated test runs, or appeared then for the first time (Figure 3E).

II. Factors altering the normal response.

One of the chief causes for unsatisfactory myograms was an inability of patients to relax dur-

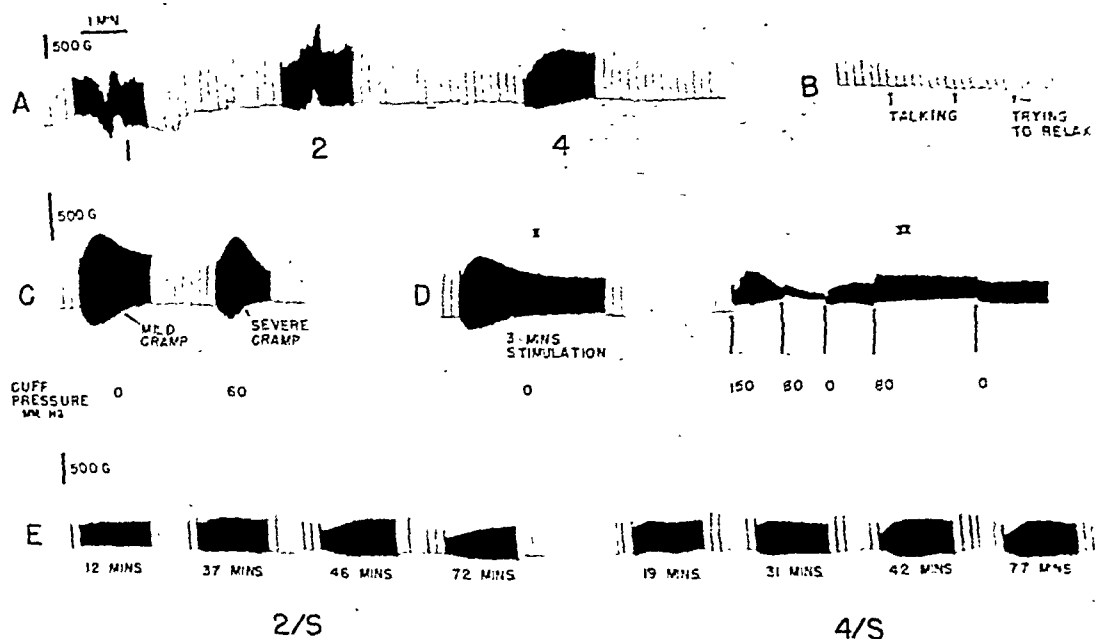


FIG. 3. MYOGRAMS ON NORMAL SUBJECTS

Explanation of figure is the same as for Figure 2. (Parts of the myogram were re-inked before being photographed.) A. Myogram of 33-year-old man with severe anxiety state. During the first 2 tests the patient was emotionally upset and almost "in tears." Note that his response is within normal limits. B. Record of a normal but moderately tense young woman during a casual conversation. Tensing of all arm muscles caused a decrease in contraction height. Stimulation rate: 1 per 10 seconds. C. Record showing influence of an inflated pressure cuff above the elbow of the arm being tested. A rapid decline in the height of contraction occurred in association with a severe cramp in the forearm. D. Normal man (same as subject B, Figure 2). D₁, normal curve; 3 minutes of stimulation at a rate of 4 per second. D₂, influences of a blood pressure cuff inflated to various levels; blood pressure of subject 114 per 74. E. Influence of prolonged testing. During a period of 1 hour and 20 minutes, 12 tests (6 at 2 per second and 6 at 4 per second) were made. Between these tests stimuli were applied every 10 seconds. Note the development of a primary dip after prolonged stimulation.

ing experiment. Figure 3A shows the effect of general muscular tension on a myographic record: the subject was in a state of chronic severe anxiety but otherwise well. Note also the effect of mental concentration on the muscle contractions (Figure 3B). This appeared to increase the general muscular tension, particularly of the antagonistic muscles, and resulted in a lessening of the contraction height. This effect sometimes became less marked at stimulation rates of 4 per second or faster, as the subjects were then less able voluntarily to control their contractions. Mild sedation with phenobarbital (60 mgm.) and assurance were of assistance, but never abolished the "emotional tension factor." Significant pain was almost constantly present during stimulation frequencies of 16 per second or faster, and

was often present at 8 per second. As a consequence many of these records were of doubtful reliability.

The character of the myogram was influenced some by the tension of the myograph (compare Figure 2B, D, and E). It was an inconstant observation that increased tension was associated with an increased potentiation. With less than ideal tension the myograph lever overswung. To avoid these difficulties the optimal tension for each subject was chosen with care and was the same during repeated tests.

Pain in the contracting muscles, accompanied by a cold and moist hand, attended the falling off of contraction which occurred toward the end of test runs at 2, 4, and 8 per second stimulations. This pain was identical to that which resulted

from ischemia. Several experiments were done in an attempt to clarify this relationship. A blood pressure cuff was applied about the upper part of the arm that was being tested. The cuff was inflated to 60 mm. Hg before delivery of tetanus. In an individual who repeatedly showed terminal falling off of the height of contractions only at a stimulation rate of 4 per second, this phenomenon occurred much more rapidly and was associated with an increased amount of pain (Figure 3C). In another experiment (Figure 3D), following a control test at 4 per second for 3 minutes, the pressure of the blood pressure cuff was raised above systolic pressure and the stimuli discharged at the same rate (4 per second). The contractions weakened rapidly, but returned slowly toward normal after complete release of the pressure. The cuff was then re-inflated to just above the diastolic pressure of the subject and the stimulation was continued at 4 per second for 2 minutes with only slight fall in the contraction height. While the stimulation was continued the cuff pressure was then released and only a barely perceptible increase in the contraction height occurred. To exclude interference of the cuff with muscular contractions, in other experiments localized pressure was applied to the brachial artery; the contraction response was the same as with the cuff.

The roles of general body anoxia and hypoglycemia were investigated. One subject breathed a 10 per cent oxygen-90 per cent nitrogen mixture until cyanosis was present. During several periods of cyanosis which lasted 10 to 15 minutes, standard tests were done. The responses were identical with those observed under normal conditions. Likewise 2 subjects who were made hypoglycemic by the administration of insulin intravenously (blood sugars reached 27 and 20 mgm. per cent, respectively) showed responses no different from those obtained under normal conditions.

III. Myograms in pathological states.

(a) *Addison's disease*: The method of myography described here was developed to aid in the evaluation of the muscular function of patients with Addison's disease being treated with various adrenal cortical hormone preparations. In general, only those who exhibited marked

weakness clinically showed significant changes in their muscle curves. Two examples of this will be presented with short abstracts of their clinical histories (1).

Patient H. J., a 55-year-old male, was maintained on large supplementary doses of NaCl (14 grams daily), from 1939 to 1943. In 1940 a right orchidectomy was performed for tuberculosis. In 1943 injections of desoxycorticosterone were begun. A tuberculous kidney was removed in 1944, and thereafter he was maintained on pellets. This patient showed a tendency to develop hypertension. Hypoglycemic manifestations occurred only during intercurrent infections. The 17-ketosteroid excretion was 3.8 mgm. daily and the B.M.R., - 18 per cent.

The patient was maintained in the hospital for 6 days on presumably adequate doses of desoxycorticosterone (1.5 mgm. every other day) and salt. On the third day of this regimen, a myogram (Figure 4A) showed slight potentiation of muscle contractions at 0.5, 1, and 2 per second test runs and a prominent primary dip followed by no potentiation at 4 per second. On the sixth day (Figure 4B) the patient complained of increasing weakness. His myograms exhibited no potentiation of muscle contractions at 0.5 and 1 per second, and only slight potentiation at 2 and 4 per second rates. A primary dip was present at both these speeds. After 3 days of treatment with desoxycorticosterone (1 mgm. daily) and 15 mgm. of corticosterone daily, the patient felt much stronger and exhibited improved muscle function. There was increased contraction height and normal muscular potentiation at 0.5, 1, 2, and 4 per second tests with considerable lessening of the primary dip at 2 and 4 per second (Figure 4C).

Patient M. N. was maintained on pellets of desoxycorticosterone acetate. She had a tendency for edema to develop when given more than 2 mgm. of hormone daily. The patient also had frequent spontaneous bouts of hypoglycemia which were prevented by daily 3-cc. injections of Upjohn's adrenal extract in oil. This latter treatment greatly improved her muscular strength. It was not possible for her to maintain her weight and strength when receiving only 3 cc. of Lipo-Adrenal Cortex daily. The 17-ketosteroid excretion was 0, and B.M.R., - 18 per cent.

She had been receiving Upjohn's Lipo-Adrenal

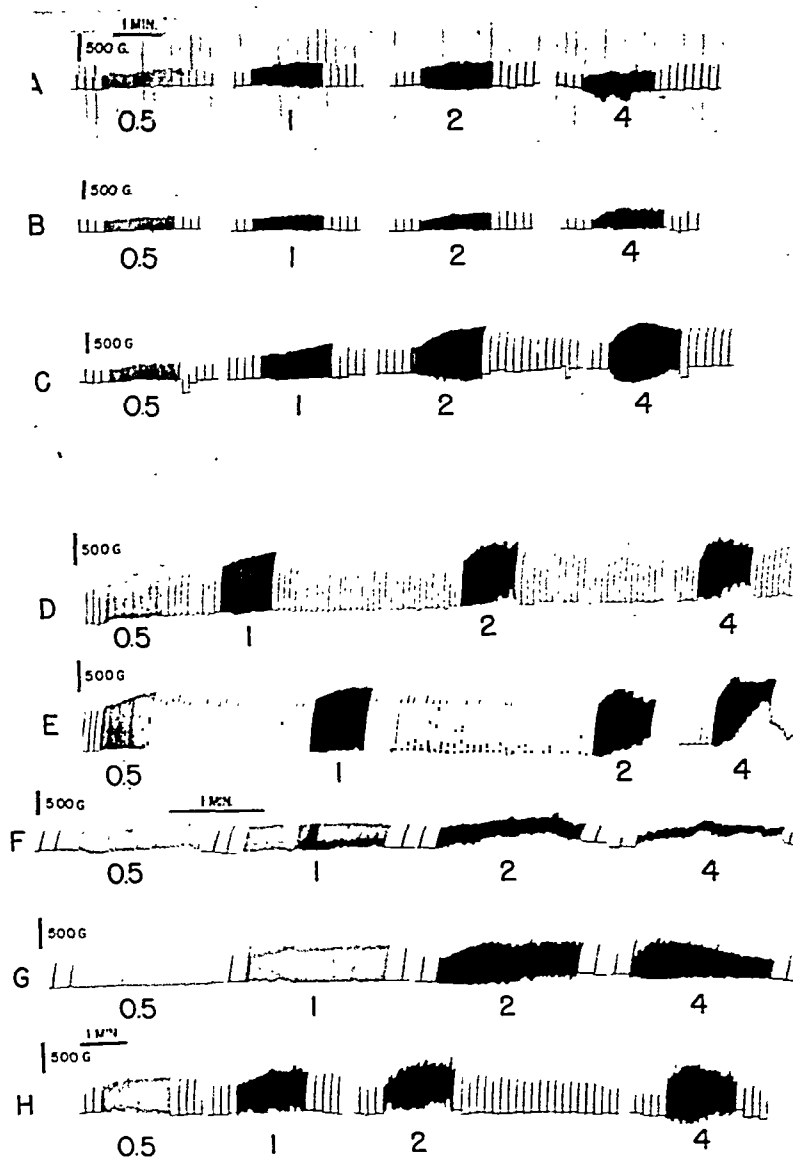


FIG. 4. MYOGRAMS ON PATIENTS WITH ADDISON'S DISEASE

Explanation of figures is the same as for Figure 2. A. H. J. Therapy: desoxycorticosterone plus salt. B. H. J. Three days later; same therapy. C. H. J. Compound A added to above treatment for 3 days. D. M. N. Therapy: Upjohn's extract and eschatin. E. M. N. Patient given only desoxycorticosterone for 3 days. F. M. N. Six hours later, after receiving 30 mgm. Compound A in oil. G. M. N. Two days later, after combined desoxycorticosterone and Compound A in oil therapy. H. M. N. Three days on combined treatment.

Cortex for several days when the myographic curves shown in Figure 4D (regarded as normal) were taken. She was then placed on 1 mgm. of desoxycorticosterone daily for 3 days, the Lipo-

Adrenal Cortex being discontinued. On this regimen she became weak and easily exhausted. A myogram (Figure 4E) showed a decrease of the contraction height near the middle of the 2

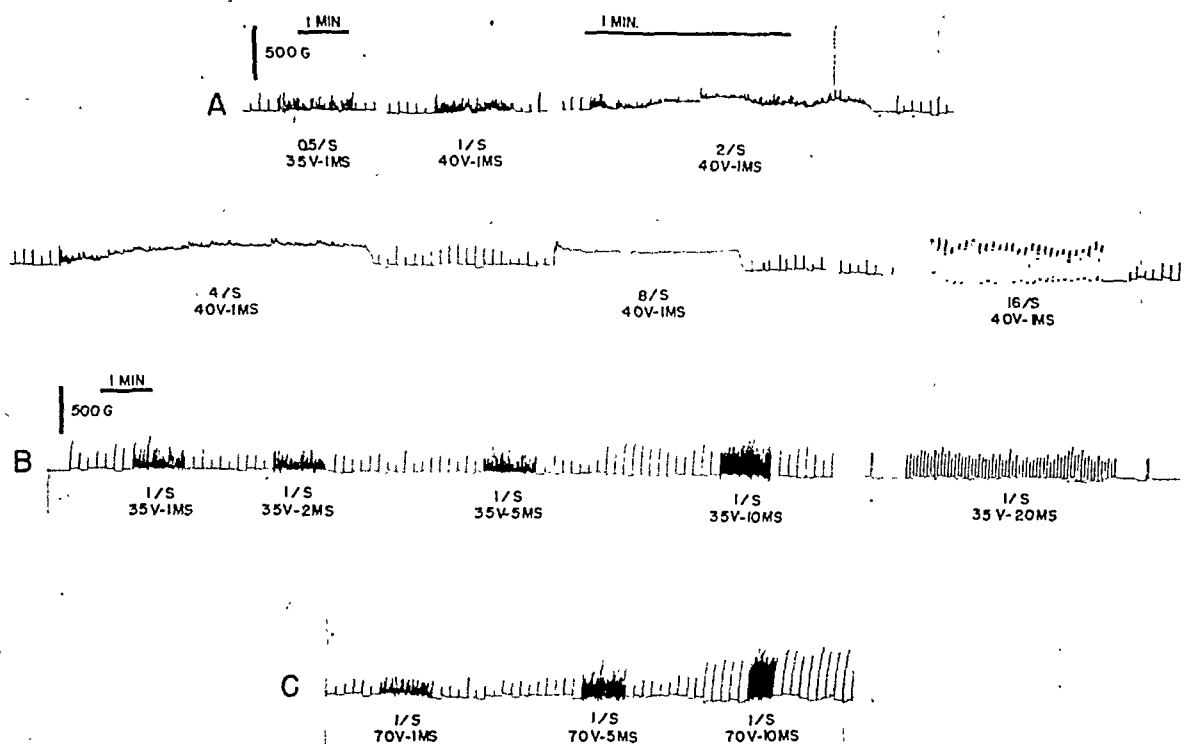


FIG. 5. MYOGRAM ON A PATIENT WITH PROGRESSIVE MUSCULAR ATROPHY

A. A 43-year-old woman with marked atrophy of both forearms. Note irregularity of muscular contractions. B. Same subject. Increasing duration of stimulation from 1 millisecond to 20 milliseconds increases height of contraction but does not eliminate irregularity of the responses. C. Same subject. Electrode applied over the flexor carpi ulnaris muscle.

per second test. In spite of an injection of 20 mgm. corticosterone given intramuscularly in oil, a repeat test 6 hours later showed even greater evidence of impaired muscle function, and this correlated with her increased weakness (Figure 4F). There was a complete absence of potentiation during test runs at 0.5, 1, and 2 per second in addition to a falling off of the contraction height at 2 per second. Three days later after receiving 1 mgm. of desoxycorticosterone and 30 mgm. corticosterone daily for 2 days, the patient felt stronger (see Figure 4G), and 3 days later very much stronger (Figure 4H). Note the increased height of single muscle contractions, and improved muscle potentiation.

(b) *Progressive muscular atrophy*: Two patients with this condition were studied. In 1 instance the atrophy in the upper extremities was mild, in the second it was severe. This latter patient had noticed the onset of weakness, fibrillations, and atrophy in the hands about 18 months earlier. The condition had been steadily pro-

gressive, and when the studies reported here were done, the atrophy and weakness of both forearms and hands were severe and frequent fibrillations of the tested muscles were observed. She could grasp a pencil with either hand and hold it for writing, but writing was accomplished largely by movements of the shoulder and elbow. There was moderate muscle atrophy and weakness of the shoulder girdles, but no weakness or atrophy of the trunk or lower extremities. Signs of pyramidal tract disease were absent.

This patient's myograms are shown in Figure 5. Figure 5A shows her response to a standard test plus 8 and 16 per second tests. The outstanding feature of this test in addition to evident weakness is the irregular height of the contractions, regardless of the stimulation speed. In Figure 5B the duration of each electrical stimulation is increased from 1 to 20 milliseconds. In spite of the general increase in the contraction height, the variation in individual contractions persisted. The single twitches in the 20-milli-

second series are notched at their peaks due to repetition of the muscle twitch. In other experiments the stimulation current was increased to 70 volts with no significant difference in the character of the muscular contractions. In Figure 5C the stimulating electrode was placed on the skin over the flexor carpi ulnaris muscle. The character of the contractions was the same as before. An additional feature in this case was a tendency for tetanus to be fused at stimulation speeds of 4 per second. At 8 per second functional fusion was produced (see Figure 5A).

The myograms of the patient with less marked atrophy showed lesser, but similar irregularities of contraction, during testing. In this patient there was no tendency to fusion of tetanus at the stimulation speed of 4 per second.

DISCUSSION

Two alterations in the myograms of normal subjects might have been considered evidence of "fatigue" and certainly indicated an altered state of muscle function. The first was a prominent dip in the myogram immediately after the beginning of a 2 or 4 per second test which was then followed by the usual potentiation of muscle contractions. This change usually became prominent, or in some subjects appeared for the first time, after repeated stimulation of the ulnar nerve over periods of an hour or longer. In correlation with this, von Euler and Swank (2) observed this phenomenon to be well developed in cats and pigeons toward the end of long experiments. These observations suggest that this change in the myogram is a manifestation of an alteration in the state of the muscle, not quickly reversible to normal.

The second change in the myogram which correlated with "fatigue" was a falling off of muscle contractions in the last 30 to 45 seconds of a 1½-minute test at 2 to 4 per second. This was also produced by ischemia of the contracting muscles. The pain in both instances was identical and it is felt that the mechanism in both instances was in large part ischemia; in the former due to the contracting muscles or to vasoconstriction incident to slow stimulation or apprehension, and in the latter to the blood pressure cuff.

The early dip in the myogram occurred in exaggerated form in Addison's disease, and it was

usually followed by a less than normal potentiation. Adequate treatment of the Addison's disease was accompanied by reversal of these changes toward normal. Like the normals, all patients with Addison's disease exhibited a falling off of the height of contraction during the latter part of a 4 per second test, and unlike the normals most of them showed this same phenomenon during 2 per second tests.

A third alteration in the myogram was observed only in patients with Addison's disease and in 2 others with thyrotoxic myopathy. It consisted of an absence of potentiation of the muscular contractions normally present during repeated stimulation. This was accompanied by severe and generalized weakness. This would appear to be a manifestation of the abnormal metabolic state present in the muscles of patients with Addison's disease since normal muscle potentiation was observed in these same muscles after treatment of the underlying deficiency. It should not be overlooked that there was a delay of several days at least between the giving of adequate treatment for the Addison's disease and restoration of a normal subjective feeling of strength and a normal muscle myogram.

The method of myography described herein detected and recorded alterations in muscle function in severe Addison's disease. In milder Addison's disease, even though subjective weakness and fatigability were complained of, the myograms were usually normal. In general it can be stated that the patient's subjective feeling of weakness or fatigue was probably a more sensitive indication of the severity of the Addison's disease than the myogram. It is of interest that neither hypoxemia nor hypoglycemia of a severe grade (yet neither severe enough to produce unconsciousness) were attended by any of the manifestations of altered muscle function described in this paper.

High frequency stimulation, as used by Odum *et al.* (3), was not employed because of the pain which it produced. It was felt by these workers that they were inducing fatigue at the neuromuscular junction. The rates of stimulation used in our experiments, in all probability, did not produce fatigue at the neuromuscular junction, but demonstrated an alteration primarily in muscle function. The work of del Pozo on cats lends support to this (4). With stimulation frequencies

of 30 per second and greater, a type of fatigue resulted which was quickly reversible to normal. This was thought to be due to transmission fatigue. Following stimulation with frequencies of less than 20 per second, the rate of recovery was slow. This was considered due to fatigue in the muscle.

The variability of the contraction height in patients with primary muscular atrophy suggests a remarkable spontaneous alteration of the sensitivity of the neuromuscular apparatus to electrical stimulation. It is unlikely that the failure of each stimulation to elicit uniform responses was due to subthreshold stimulation, since very high voltages were tried without altering this character of the myogram. The variation may be explained, however, by the observation of Bourguignon (5) that the chronaxie of involved muscles in patients with amyotrophic lateral sclerosis is at first reduced and later increased. Whereas the more irritable units respond to each electrical stimulation, the less irritable ones frequently fail to respond. The peripheral nerve and its cell body as well appear also to be abnormally irritable in this disease since novocainization of peripheral nerves progressively nearer to the muscles which they innervate diminishes progressively the number of fasciculations in these same muscles, without completely abolishing them until the novocaine is injected into the fasciculating muscle (Swank and Price, [6]). Preceding (Grund, [7], Shelden and Woltman, [8]) and more recent work (Odom, Russel, and McEachern, [3], Forster, Borkowski, and Alpers, [9]), showing that fibrillations still occur in muscles deprived of their motor innervation by novocainization or surgical incision, confirms the observation that the stimuli giving rise to muscle fasciculations can arise in the distal part of the peripheral nerve, but does not establish this region as the only possible point for their origin.

A possible relationship exists between the myograms, and the predominating sizes of motor nerve fibers in normals and in patients with amyotrophic lateral sclerosis. It has been shown that normal ventral spinal roots contain 2 general classes of nerve fibers, small and large (Kiss and von Mihalik, [10], Eccles and Sherrington, [11], Häggqvist, [12], and Swensson, [13]). In amyotrophic lateral sclerosis the large fibers degenerate first, leaving the small fibers relatively intact (Wohlfart

and Swank, [14]). In patients with severe muscle atrophy very few large fibers may be left. If the small and large motor nerve fibers have different functions then one wonders which part of the myogram from the patient with progressive muscular atrophy was due to the few remaining large fibers, and which was due to the large number of intact small fibers. Marked weakness was obvious. The answers to other questions are not clear, however. Was the remaining power due to small fibers or to the few remaining large fibers? Was the variation in strength of contraction related to large or small fiber function? Was the tendency to fusion of tetanus at the stimulation frequency of 4 per second due to a predominance of small fibers in the motor nerves?

It is of some interest that a tendency to fusion of tetanus was observed in most normal patients at stimulation frequencies of 8 per second, and in some instances (Figure 2A) functional fusion seemed to have developed by the end of the test. When stimulated at 16 per second a sustained fusion of tetanus was observed in all patients.⁴

SUMMARY AND CONCLUSIONS

To record alterations in muscle function in humans, a method of myography commonly used in animals has been employed. In normal subjects slight changes in the myogram were detected which correlated with fatigue. In patients with severe Addison's disease and in others with thyrotoxic myopathy these changes were exaggerated, and an absence of the muscle potentiation normally present during repeated stimulation was observed. These alterations were restored to normal in patients with Addison's disease by adequate treatment. In other patients with progressive muscular atrophy a marked variation in the height of muscle contraction was observed. The factors which normally influence the character of the myogram are described. Pain, due to electrical stimulation, of the ulnar nerve, is the most important of these factors, and causes a number of unsatisfactory myograms.

⁴ Beginning fusion of tetanus at stimulation frequencies of less than 20 per second is illustrated several times in a paper by Dr. Derek Denny-Brown entitled "Interpretation of the Electromyogram" which is now in press with the Archives of Neurology and Psychiatry.

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THE EFFECT OF TETRAETHYLAMMONIUM ON THE SMALL BOWEL OF MAN

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Present evidence indicates that tetraethylammonium ion blocks autonomic effector systems at their ganglia. Injection of the drug prevents the passage through the superior cervical ganglion of nerve impulses to the nictitating membrane of the cat (1). Similarly, there occurs inhibition of the effect on the decentralized heart of preganglionic stimulation of either vagal cardio-inhibitory fibers or of the cardio-accelerators (2). The sharp fall in arterial blood pressure accompanying intravenous injection results from ganglionic blockade of the sympathetic vasoconstrictor nerves (2).

The action of tetraethylammonium has been extensively studied in man by Lyons and his associates (3 to 5). After single intravenous injection of 200 to 300 mgm. of the chloride salt there is a fall in blood pressure and a rise in heart rate. These values have usually returned to normal in 10 to 15 minutes after injection. There is an accompanying sensation of tingling and warmth of the face and extremities and sometimes drowsiness and momentary paralysis of accommodation. Serious reactions have not been reported. After giving nearly 100 injections of the drug we observed 1 marked reaction. This patient (Case 4) had an alarming and persistent tachycardia and fall in blood pressure which lasted for 5 days (6).

The action of tetraethylammonium on the gastrointestinal tract has received relatively little attention. Lyons has reported that after intramuscular injection marked diminution in contractions occurs. His patients with peptic ulcer have received dramatic but temporary relief from ulcer pain. Cessation of gastro-intestinal motility has been observed fluoroscopically after ingestion of a barium meal, and abdominal cramps and diarrhea have been abolished. Gastric acidity is said to be diminished (4).

This report concerns the effect of tetraethylammonium ion on the intestinal tract of 8 patients as observed by the multiple balloon technique. Three patients were hypertensive. Of these 1 had a duodenal ulcer and another had had a lumbo-dorsal sympathectomy 4 years previously without striking change in her blood pressure. Three of our patients had intractable abdominal pain, presumably intestinal in origin. One had gastritis and 1 had rheumatoid arthritis.

In 2 patients the amount of balloon distension of the intestine essential to elicit beginning pain was determined before and during the action of the drug. The purpose of this observation was to find out whether tetraethylammonium affects the sensory impulses arising in the intestine and thereby relieves intestinal pain.

METHOD

Patients were studied in the fasting state. The motility of the duodenum and jejunum was recorded by means of a 4-channel tube. Balloons were separated at 4-inch intervals, each balloon being connected to a separate U-shaped water manometer (7). Ink writers from each manometer were placed so that contractions were recorded simultaneously in a vertical line. The balloons and tubes were introduced under fluoroscopic control. Each balloon was of the same size and was filled with 20 cc. of air. This was found to be sufficient to fill the intestinal lumen but was well below the amount necessary to elicit pain from stretching of smooth muscle fibers. Chest movements were recorded from a blood pressure cuff taped around the chest wall. The kymograph moved at a rate of about 3 cm. per minute. Blood pressure and pulse were recorded at 2- to 3-minute intervals for 15 minutes before the injection and for 30 to 45 minutes afterward. Tetraethylammonium was administered intravenously at a rate of approximately 200 mgm. per minute. It should be noted that in tetraethylammonium content 390 mgm. of the chloride salt is approximately equivalent to 500 mgm. of the bromide salt. During and following the injection, signs and symptoms which developed were noted. Adrenalin and neosynephrin were always on hand in case of untoward reactions.

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TABLE I

Case No.	Diagnosis	Dose	Onset of action after start of inject:	Changes in intestinal activity		Partial return of activity		Full return of activity		Effect on symptoms
				Tone	Contractions*	Tone	Contractions*	Tone	Contractions*	
1. 534560	Rheumatoid arthritis	mgm. 500	seconds 30	Decrease	Decrease only	30	30	45	45	
2. 538260	Intractable pain	500	45	Decrease	Brief decrease only	Only 15' tracing after injection				
3. 114951	Hypertension; lumbodorsal sympathectomy 4 years ago	300	60	Decrease	Absent†	30	20	75 (Return not yet complete)	75	
4. 370990	Intractable pain	230	45	Decrease	Absent†	40	39	65 (Return not yet complete)	65	Pain disappeared with cessation of contractions
5. 190980	Duodenal ulcer and hypertension	400	45	Decrease	Absent†	35	35	55	55	
6. 537908	Hypertension	300	45	Decrease	Absent†	55	30	60 (Return not yet complete)	60	
7. 175870	Gastritis	400	35	No decrease	Absent‡				56 (Sudden and complete return)	
8. 544965	Intractable pain	300	None	No decrease	No decrease					
	(45 min. later)	400	120	Decrease	Decreased and absent§	35	43	53	51	Pain disappeared

* Refers to both ring-like contractions and propulsive movements.

† Contractions eliminated in all 4 balloons.

‡ Only 2 balloons in duodenum. Contractions eliminated.

§ Only 3 balloons in duodenum. Contractions eliminated in 2, decreased in the third.

The threshold of beginning intestinal pain was obtained by inflating the balloon in the duodenum at a rate of a 4- to 6-cm. rise in water pressure per second until pain was first felt. The average of 5 consecutive determinations was taken to be the pain threshold level. The determinations were repeated 5 minutes after the drug had been given.

RESULTS

Table I contains a summary of the results obtained in the 8 patients. In all except Case 8 contractions began to decrease in 30 to 60 seconds from the start of the injection. In 60 seconds no more than 200 mgm. of the salt had been given. In Case 8 abdominal muscular contractions which accompanied the patient's pain obscured the intestinal movements as recorded by the balloons.

After receiving 300 mgm. of the chloride salt this patient reported practically no relief of pain and showed only slight decrease in contractions of the abdominal muscles. Forty-five minutes later she received a second injection of 400 mgm., at the completion of which she said that she was free of pain. Abdominal muscle contractions almost completely ceased. For the next 43 minutes no movements were recorded from the balloons in her duodenum and jejunum other than artifacts resulting from coughing, body movements and respirations. This fact suggested that intestinal contractions disappeared following the second injection.

In 5 out of 8 patients, including Case 8 following her second injection, ring-like contractions as well

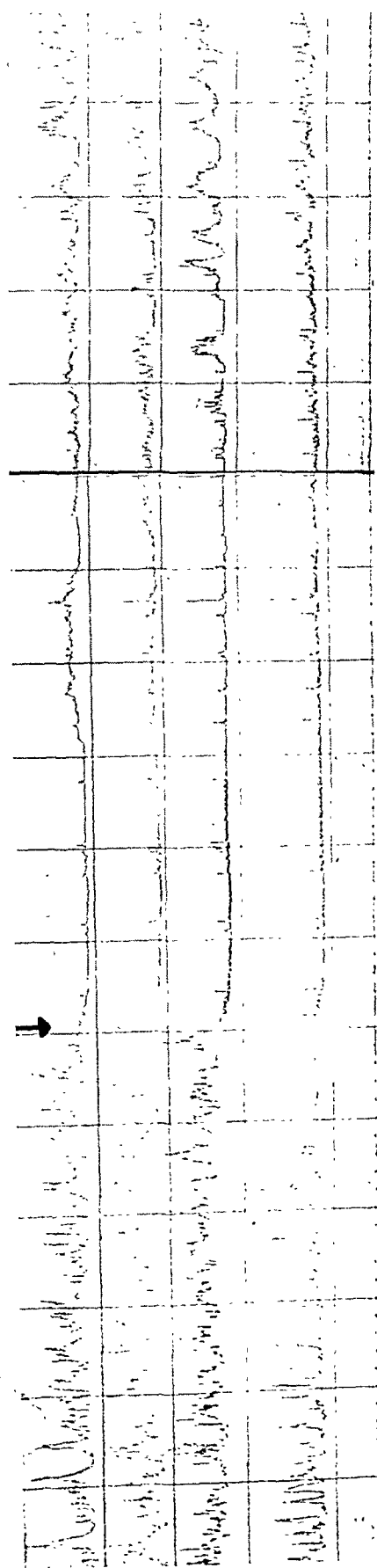


FIG. 1. TRACING OF THE UPPER SMALL INTESTINE

This shows the fall in the baseline and disappearance of contractions 45 seconds following the beginning of the injection of tetraethylammonium 400 mgm. I.V. Ring-like contractions and propulsive waves were absent except in the top tracing for 35 minutes, at which time both types of contractions began to return along with a beginning elevation of the baseline. Intestinal activity was back to the pre-drug level 55 minutes following the injection. The top, or No. 1, tracing is from the distal balloon and the second, third and fourth tracings are recordings of activity at successive proximal levels. Chest movements are recorded at the bottom tracing. The vertical lines represent 5-minute intervals.

as propulsive movements were abolished (Figure 1) much as they were by atropine (Figure 2). The ring-like contractions are represented by the regularly occurring waves as shown in the figures. Propulsive movements are represented by the sustained elevation of the base of the contraction waves. When the contractions disappeared the baseline of the tracing fell, suggesting a decrease in smooth muscle tone. Certain technical factors made it difficult to quantitate this fall. Transmitted pressure from the abdominal wall contributed to the height of the baseline. Also, distension of the gut by the balloon itself increased contractions and raised the baseline. Since neither of these factors could be measured we are unable to interpret baseline changes in quantitative terms, but only to indicate trends. In all patients except 1 the disappearance of contractions was accompanied by a fall in the baseline of the tracing.

Resumption of activity began between 20 and 43 minutes following the injection, although the contractions did not appear from all 4 balloons simultaneously. In Case 4 the failure of return of activity before 40 minutes might not have been accountable by the effect of tetraethylammonium alone. By this time this patient had already received large doses of epinephrine and neosynephrin intravenously. In 3 patients who were studied until there was complete return of activity the contractions were back to normal in 45 to 56 minutes. Three patients were studied for 60, 65 and 75 minutes, respectively. At these times their contractions had not entirely returned to normal. Three of our patients were hospitalized for study of severe abdominal pain of obscure origin. Two of these patients were complaining of this pain at the time of the injection. In both there was a disappearance of pain following the injection with simultaneous abolition of contractions in 1 patient and marked reduction of contractions in the other.

In the 2 patients whose thresholds for beginning pain were measured by balloon distension, the number of centimeters of water pressure essential to elicit pain was unchanged 5 minutes after the drug had been given. The first patient's pre-drug threshold averaged 70 cm. of water pressure and post-drug threshold, 72 cm. The second patient's pre-drug threshold averaged 84 cm. of water pressure. After the drug the threshold level averaged 80 cm. of water pressure.

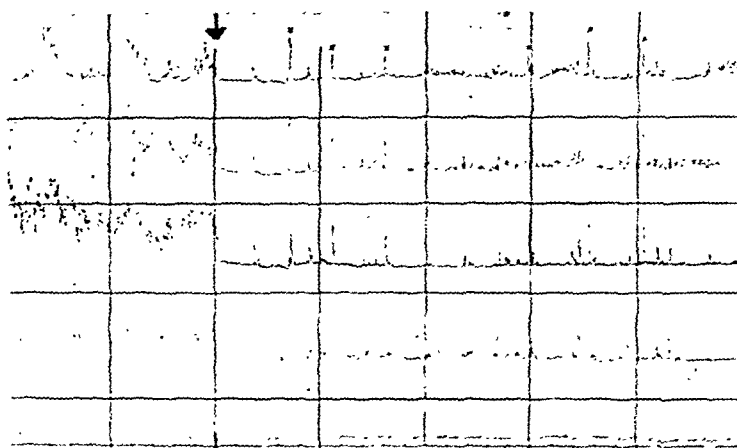


FIG. 2. EFFECT OF 0.6 MGML. OF ATROPINE SULFATE I.V.

The same subject as in Figure 1 was given 0.6 mgm. of atropine sulfate I.V. over a 30-second interval. At the end of injection there was a fall in the baseline, a disappearance of propulsive waves and abolition of ring-like contractions. The movements noted following the injection are artifacts due to body movements and respirations. From 10 to 15 minutes following the injection there was a slight return of ring-like contractions. It is apparent from this tracing that the effect on intestinal motility is much the same as that following the injection of tetraethylammonium. The balloon arrangement and positions are the same as in Figure 1.

DISCUSSION

Tetraethylammonium causes an immediate cessation, or marked decrease, of motility of the upper small bowel. This effect is more prolonged than the fall in blood pressure or rise in pulse which the drug induces. It is possible that buffer reflexes for the circulation are more highly developed and account for the more rapid return of blood pressure to normal. Within the limitations of our method the effect on the intestine is identical to that of atropine.

The control of the activity of the small bowel is exceedingly complex and poorly understood. Not only is this activity under the partial control of sympathetic fibers and the vagus but the myenteric plexus of Auerbach, the plexus of Meissner, and the smooth muscle fibers themselves contribute to the regulation of intestinal activity. If tetraethylammonium blocks vagal and sympathetic impulses to the gut at the respective ganglia, as one might expect from analogy to results obtained in other autonomic effector systems, then injection of the drug might be expected to produce effects similar to those obtained by section of autonomic fibers. In the dog, rabbit and cat under alcohol-chloro-

form-ether anesthesia soon after a combined splanchnicectomy and vagotomy, the bowel is found to be quite active (8, 9). Pendular movements as well as progressive ring-like contractions are still present. Unless the splanchnics are cut the small intestine is found to be in a profound state of inhibition when the abdomen is opened, and this inhibition is released only when the splanchnic fibers are sectioned. Conversely, section of the vagi causes no further change in the contractions. In the combined operation, stimulation of the peripheral end of the cut splanchnics leads to complete inhibition whereas prolonged and repetitive stimulation of the peripheral end of the cut vagus is followed by enhanced contraction of the small bowel, except only that the first part of the duodenum responds more quickly and seems to be more directly under vagal control. Such vagal effects can be blocked by small doses of nicotine but not by large doses of atropine in animals. However, in man large doses of atropine continue to decrease intestinal contractions following vagus resection. Procaine block of the lumbar sympathetic ganglia supplying the gut in man leads to augmented contractions of the small bowel and colon (10).

Three days after transthoracic vagotomy the ring-like contractions of the bowel are decreased (observation in 1 subject). This effect may in part be due to trauma of operation (10). Observations immediately postoperative have not yet been made. *In vitro*, the longitudinal contractions of the rabbit intestine are enhanced by tetraethylammonium (11), but no studies on the circular component have been made.

These considerations suggest that the mechanism of action of tetraethylammonium on the small intestine is not solely that of an autonomic blocking agent. Perhaps this drug has an additional action on the intrinsic neuronal structure or on the smooth muscle itself which are responsible for the continued activity following surgical denervation.

The fact that the thresholds for intestinal pain elicited by balloon distension were unchanged following the administration of tetraethylammonium suggests that this drug has no significant action on the sensory innervation of the intestine. Its pain-relieving effect must, therefore, depend on the anti-spasmodic action.

If continued use demonstrates its reasonable safety, tetraethylammonium may have diagnostic value in implicating smooth muscle spasm as responsible for the features of certain cases of obscure abdominal pain. Its value as a relaxing agent in such conditions as ulcerative colitis and intractable peptic ulcer is limited, at least at present, by its brief duration of action.

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THE PROTHROMBIN RESPONSE TO THE PARENTERAL ADMINISTRATION OF LARGE DOSES OF VITAMIN K IN SUBJECTS WITH NORMAL LIVER FUNCTION AND IN CASES OF LIVER DISEASE: A STANDARDIZED TEST FOR THE ESTIMATION OF HEPATIC FUNCTION¹

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There are a multiplicity of tests for liver functions, each depending upon a particular function of the liver. There has been growing recognition at the response in prothrombin time to the administration of vitamin K is of aid in the evaluation of hepatic function. The procedures heretofore employed to estimate the prothrombin response have lacked uniformity and, as a consequence, conflicting opinions of its significance and reliability have been expressed. Need exists for further study in both normal and abnormal subjects, and comparison with other liver function tests to establish its status as an index of at least one particular function of the liver. The present study deals with a standardized, sensitive method for the estimation of the prothrombin response to the administration of vitamin K. Our results show that the ability to maintain a normal prothrombin level is a function of the liver apt to be affected before others are sufficiently disturbed to permit clinical detection. For this reason, the response of the liver in prothrombin production to the parenteral administration of large doses of synthetic vitamin K is a sensitive indicator of hepatic function.

The use of plasma prothrombin levels as an indicator of the ability of the liver to elaborate prothrombin was originally studied by Stewart and Rourke (1) and pursued subsequently by numerous investigators, (2 to 5), all of whom employed whole plasma for prothrombin assay. Warner, Brinkhous and Smith (6) in dogs and Pohle and Stewart (3) in man, introduced the response of plasma prothrombin levels to orally administered

vitamin K derivatives as a test of liver function. They demonstrated that the prothrombin level sometimes failed to return to normal after the ingestion of vitamin K. In a few cases, the prothrombin level became even further reduced following the vitamin K ingestion. The results of these studies need reinvestigation in the light of recent findings that the prothrombin assay techniques utilized were not sufficiently sensitive to detect fine alterations (7, 8).

In diseases such as biliary obstruction or diarrhea interfering with the absorption of vitamin K, it cannot be easily decided whether an accompanying hypoprothrombinemia is referable to liver insufficiency or to failure to absorb the vitamin. Kark and Souter (9) showed that patients with hepatic disease having prothrombin deficiency are not able to increase their prothrombin level to normal following parenteral administration of 2-methyl-1, 4-naphthoquinone. This was demonstrated, also, by Andrus and Lord (10) using 2 mgm. doses of the substance intramuscularly, and by Seligman, Hurwitz and co-workers (4) using 10 to 20 mgm. doses intravenously of synthetic vitamin K or the sodium salt of its sulphuric ester. It is interesting to note that although Seligman and Hurwitz (4) failed to comment on it, some of their patients with liver disease also exhibited further reduction in the prothrombin level after the medication. In these studies, also, the fact that the methods used were, for the most part, too insensitive to reveal alterations in plasma prothrombin levels of low magnitude needs emphasis (7, 8).

Introduction of the diluted (12.5 per cent) plasma method of estimation of prothrombin time

¹ from the Blood Transfusion Association and the Hoffman-La Roche Company.

greatly increased the sensitivity of the procedure (7, 8). Subsequently, Shapiro and Richards (11, 12) reported on the plasma prothrombin levels and the response to parenteral administration of synthetic vitamin K (Hykinone) in liver disease using this procedure. In experiments on dogs, in which liver damage had been produced by the administration of CCl_4 , it was found that the prothrombin level of the diluted (12.5 per cent) plasma was at least as frequently an indicator of hepatic disturbance as bromsulphalein retention. Their studies of 23 cases of liver disease in man, revealed moderate or marked reduction of the resting prothrombin level in 20 cases in which the reduced prothrombin level failed to return to normal following the intravenous administration of Hykinone. In some cases, moreover, a temporary reduction of a previously normal prothrombin level to abnormal levels occurred. In other patients, further depression of a previously low prothrombin level occurred.

The present study, presented in its earlier stages before the New York Pathological Society (13), is an extension of the study of Shapiro and Richards (11, 12) embodying the principle of imposing a load on the liver by the parenteral administration of large doses of vitamin K. It is the same principle that is utilized in other liver function tests such as bromsulphalein retention, bilirubin clearance, glucose tolerance, and galactose tolerance tests. This reaction to injection of vitamin K is correlated in patients with hepatic disease and in normal controls with the results of other liver function tests and liver biopsy or post-mortem findings wherever possible. Evidence is presented that the sensitivity of this test for a specific known physiologic function of the liver makes its incorporation into a battery of liver function tests highly desirable.

PROCEDURE

The resting level of prothrombin was obtained by the diluted (12.5 per cent) plasma method on several days prior to, and on the day of the initial intravenous administration of synthetic vitamin K (Tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester [Synkayvite]²). Normal plasma prothrombin time of diluted (12.5 per cent) plasma was $39.5 \text{ seconds} \pm 2.5 \text{ seconds}$

(7, 8). Whole plasma prothrombin times, estimated simultaneously, are not reported in the present study because the methods lack sensitivity and fail to eliminate the effect of natural anticoagulants and interfering substances, and because the results were not of adequate significance. At the beginning of the investigation, 38 mgm. of synthetic vitamin K (Synkayvite) was given intravenously on 4 successive days after the resting level had been determined. Later in the study, the daily dose was increased to 76 mgm. of Synkayvite. This is the dose now used. The prothrombin time was then estimated daily during the course of the test period and for several days following the last dose of vitamin K. Estimations were made in duplicate and preferably on freshly drawn fasting samples. Lipemic plasmas interfere with the accuracy of the prothrombin estimation (14).

In all subjects the results of the vitamin K tolerance test were correlated wherever feasible with bromsulphalein retention, cholesterol and esters, albumin and globulin, cephalin flocculation, icteric index and Van den Bergh reaction. There were 2 periods during the course of this study when bromsulphalein was withdrawn from use for investigation by the manufacturers, because of marked reactions which followed its administration. No untoward reaction to the dose of Synkayvite employed appeared in any of the subjects studied.

The methods used in performing the other liver function tests in this study were standard procedures. The bromsulphalein tests were made by administering 5 mgm. of bromsulphalein per kilogram of body weight, and taking a 5- and 30-minute blood specimen. Any retention of over 10 per cent bromsulphalein at the end of $\frac{1}{2}$ hour was considered abnormal. The cholesterol and ester determinations were made using the Bloor technique. Cephalin flocculation tests were performed using Difco Cephalin and reading at the end of 24 and 48 hours.

RESULTS

One hundred and thirteen tests were made on 110 individuals. Of these, 47 vitamin K tolerance tests were interpreted as negative, 9 doubtful and 57 positive.

Negative tests: A negative test is defined as one in which there is either: (Figure 1A) no significant rise in the prothrombin time from a previously normal resting level (*i.e.*, all values remain within the normal range) following the administration of test doses of synthetic vitamin K, (B) a return of prothrombin time from hypoprothrombinemic levels to normal levels, (C) a reduction in prothrombin time from normal levels to hyperprothrombinemic levels. In the negative series the resting level was usually 42 seconds or less, and the maximum prothrombin time reached during the test was 45 seconds or less with return toward

² Synkayvite supplied by Hoffman-La Roche Company, Nutley, N. J.

NEGATIVE TESTS

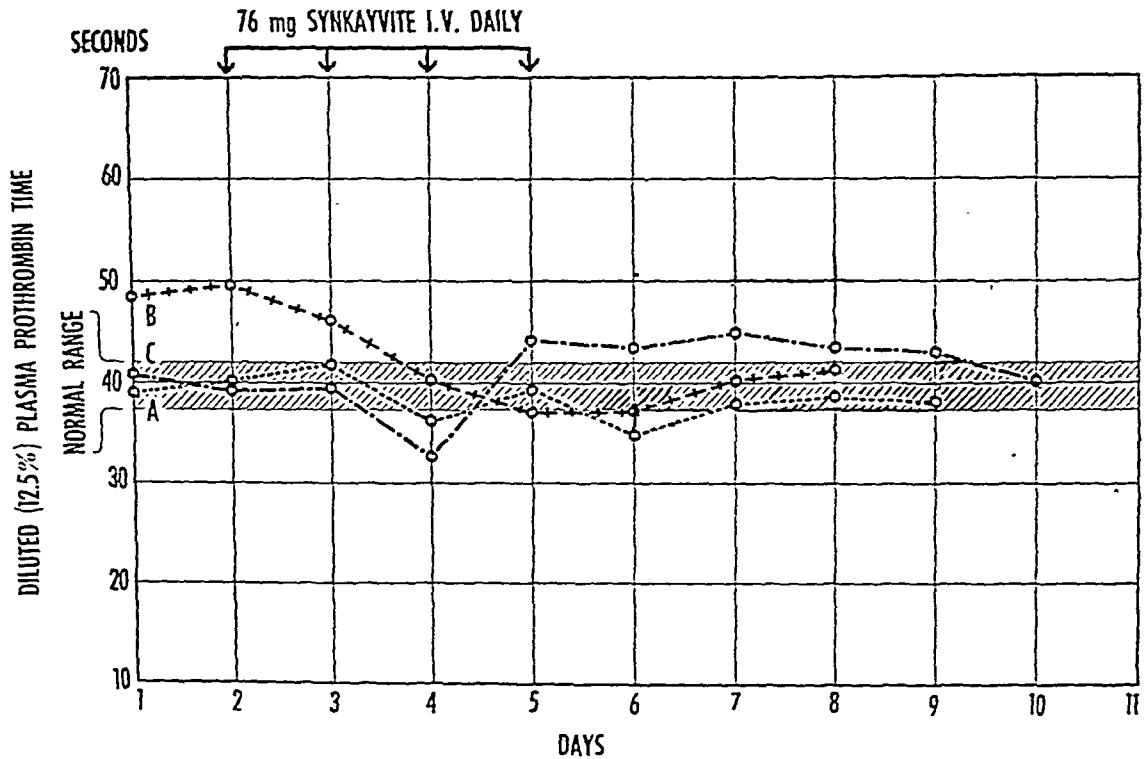


FIG. 1. THREE TYPES OF NEGATIVE TESTS

Curve A remains within the normal range throughout the period of the test. Curve B: initial hypoprothrombinemia with restoration to normal after administration of vitamin K. This is typical of nutritional vitamin K deficiency. Curve C: temporary hyperprothrombinemia after the large doses of vitamin K.

the resting level of the prothrombin time following the completion of the test. Of the 47 negative cases 35 started with resting prothrombin times of 42 seconds or less. Twelve cases started with resting times higher than 42 seconds which responded promptly to the parenteral administration of vitamin K by restoration to normal. This is the typical response seen in vitamin K deficiency states.

Positive tests: A positive test is defined as one in which there is either (Figure 2A and B) a failure to lower an abnormally prolonged prothrombin time to normal limits during the test period, (C) a further elevation of an already prolonged prothrombin time, or (D) a transient rise in prothrombin time from a previously normal value to more than 47 seconds in one or more determinations during the test period.

Out of 57, 11 cases started with normal resting

prothrombin times (12.5 per cent plasma), and 46 started with abnormal prothrombin times.

In all of these patients, the vitamin K tolerance test was positive. If the resting prothrombin time had been used alone as a test for liver function, 20 per cent of the cases would have been interpreted as showing no impairment in the hepatic function.

Furthermore some patients with prolonged pre-testing times due to deficiency states may have no impairment of liver function. It is realized that it is a somewhat arbitrary limit for bio-assay technique to establish the dividing point between normal and abnormal responses to the administration of vitamin K at 45 seconds. For this reason, a certain degree of flexibility should be followed in interpreting results several seconds over 45 until further experience shows that this level is completely justifiable. Results between 45 and 47

POSITIVE TESTS

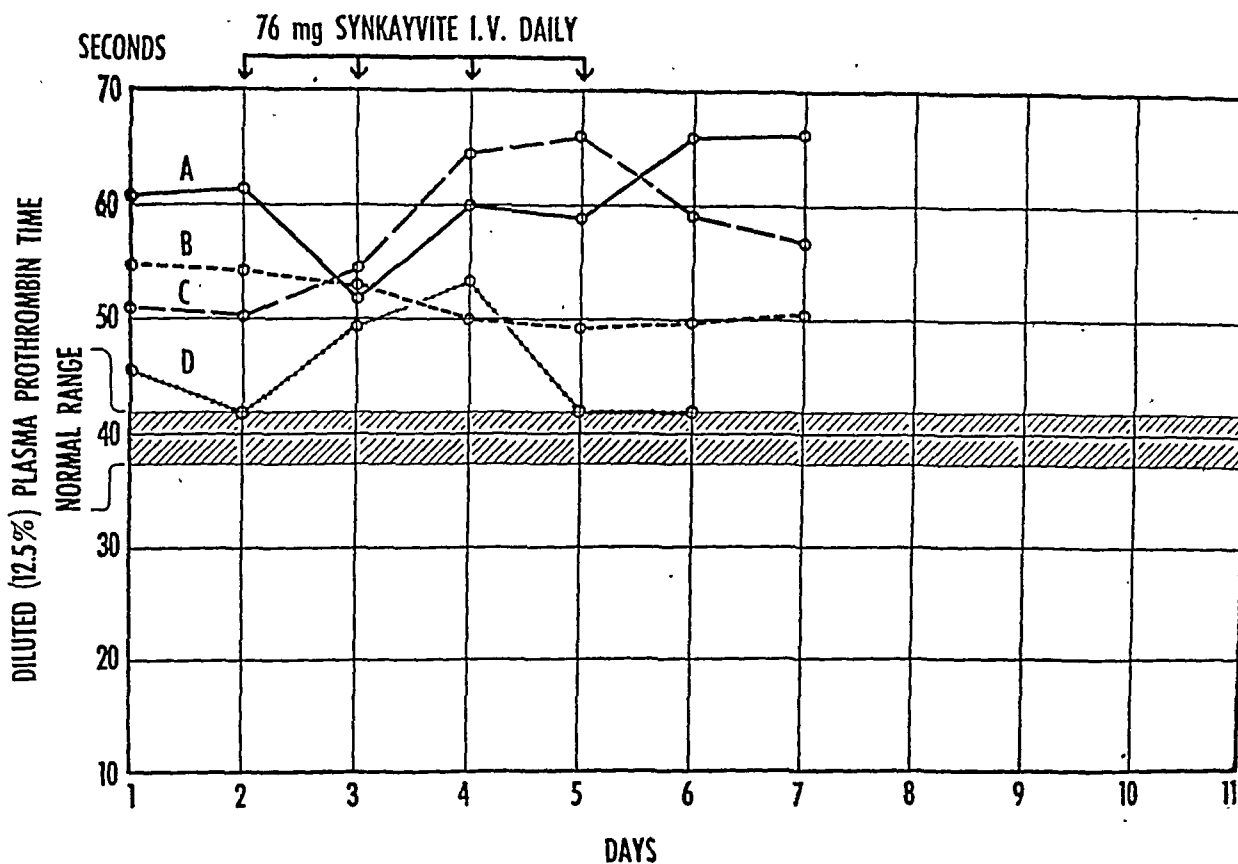


FIG. 2. POSITIVE TESTS

Curve A: initial hypoprothrombinemia with partial restoration toward normal following vitamin K administration followed by further prolongation beyond the resting level. Curve B: initial hypoprothrombinemia unaltered by vitamin K. Curve C reveals further extension of prothrombin time promptly following vitamin K administration. Curve D: normal resting level of prothrombin followed by temporary hypoprothrombinemia after vitamin K administration and eventual restoration to normal.

seconds have therefore been classified as doubtful.

The type of response to parenteral administration of vitamin K and/or its degree has not been used to date as an aid in judging prognosis.

ANALYSIS OF RESULTS

The cases have been arranged from the viewpoint of the presence or absence of liver disease on the basis of the clinical diagnosis. Cases 1 through 36 include a group of subjects, with varying diagnoses, none of whom showed any clinical evidence of liver disease. The maximum prothrombin time reached during the course of the test did not exceed 45 seconds in any of the patients in this group. Two cases, one with a maximum prothrombin time of 44.8 and the other with a maximum prothrombin time of 45.0, were classified as doubtful. Neither of these 2 cases

showed abnormal BSP retention. In the remaining 34 cases in this group of 36 patients, the vitamin K tolerance test yielded results interpreted as negative. Bromsulphalein retention was tested in 29 of these cases, with less than 10 per cent retention after $\frac{1}{2}$ hour in 25 cases, and 10 per cent or more in 4 cases. In the 4 cases where more than 10 per cent of the injected BSP was retained, the largest amount retained was 14 per cent. None of these 4 cases showed clinical evidence of liver disease.

Cases 37 through 48 consist of a group of patients where the maximum prothrombin time reached during the test also did not exceed 45 seconds. The results of the vitamin K tolerance test were interpreted as negative in these 12. Included in this group was 1 patient who showed cardiac cirrhosis on postmortem examination. The

BSP retention and cephalin flocculation tests were normal. Two patients in this group have amyloidosis and both showed negative BSP retention and cephalin flocculation. Another case showed, on postmortem examination, a carcinoma of the tail of the pancreas with a small number of metastases to the liver, too insignificant to have interfered with hepatic function. The BSP retention test was negative, and the cephalin flocculation test was 2 plus. One case was believed to have a fatty liver. This patient showed 14 per cent BSP retention after $\frac{1}{2}$ hour, a 4-plus cephalin flocculation test and a negative vitamin K tolerance test. This is the only instance in this group of 12 patients where the vitamin K tolerance test was negative despite clinical evidence of liver disease as revealed by other liver function tests. One case, despite the absence of clinical evidence of liver disease, showed an abnormal BSP retention, a cephalin flocculation of 2 plus and a negative vitamin K tolerance test. In summary, in this group of 12 patients, there were 5 with clinical evidence of liver disease in all of whom the vitamin K tolerance test was negative. In only 1 of these 5 could any evidence of impaired liver function be found using other liver function tests. It seems quite reasonable to assume that the patient with a small amount of metastases to the liver had no impairment of hepatic function. The lack of evidence of impairment of hepatic function in the 2 patients with amyloidosis is not unusual for this disease.

Cases 49 through 56 include a group of 8 cases in whom the maximum prothrombin time reached during the test ranged from 45 to 47 seconds. The vitamin K tolerance test has been interpreted as doubtful in this group, though there is reason to believe from the clinical diagnoses that the results of the vitamin K tolerance test probably belong in the positive group. It is apparent that a dividing line between positive and negative vitamin K tolerance tests establishes itself somewhere within the range of 45 to 47 seconds. For the present, until more data are collected, we will interpret results that fall within this range as doubtful and requiring further investigation. There were 5 cases in this group of 8 patients where there was definite clinical evidence of liver disease with positive BSP tests in 2 of the 5 cases. Two others showed 3-plus cephalin flocculations.

The remainder of the patients, cases 57 to 113,

all showed maximum prothrombin times exceeding 47 seconds. There were 7 patients with maximum prothrombin times ranging from 48.2 to 49.4 seconds. Four of these patients have unequivocal clinical evidence of liver disease. Of the remaining 3 cases, 1 was a patient with chronic congestive failure and persistent hepatomegaly; another was a patient with pernicious anemia; the third was a patient in whom no clinical evidence of liver disease was apparent but who showed both positive BSP and Vitamin K tolerance tests.

Cases 64 through 99 include a group of 36 cases in whom the maximum prothrombin time reached during the vitamin K tolerance test ranged from 50 to 60 seconds. Twenty-six of the patients in this group showed definite clinical evidence of liver disease. The remaining 10 patients had no evidence of specific liver disease. Five were severely anemic; 1 suffered from nutritional deficiencies with moderate anemia; and 3 showed no clinical evidence whatsoever of liver disease but were over 75 years of age. The finding of impaired liver function in the aged in the absence of clinical evidence of any specific type of hepatic disease has been reported previously (15). The remaining patient was a member of the hospital staff who was studied for control purposes, and in whom the results of vitamin K tolerance test were abnormal on 2 separate occasions. There was no history of any type of liver disease in this subject although he had served recently in the armed forces in areas where infectious hepatitis was epidemic. The only positive clinical finding was enlargement of the liver, palpable 2 fingers below the costal margin. All other liver function tests were negative, though the icteric index was 11.2 units. We consider this as a false positive vitamin K tolerance test.

The last group of 14 patients (cases 100 through 113) include those in whom the maximum prothrombin times reached during the course of the test ranged from 60 to 155 seconds. All of these cases disclosed definite clinical evidence of liver disease.

In summary, the 113 cases in whom the vitamin K tolerance test was correlated with clinical findings and other liver function tests showed the following (Figure 3): Of 43 cases with no clinical evidence of liver disease, the vitamin K tolerance test was interpreted as negative in 42 and doubt-

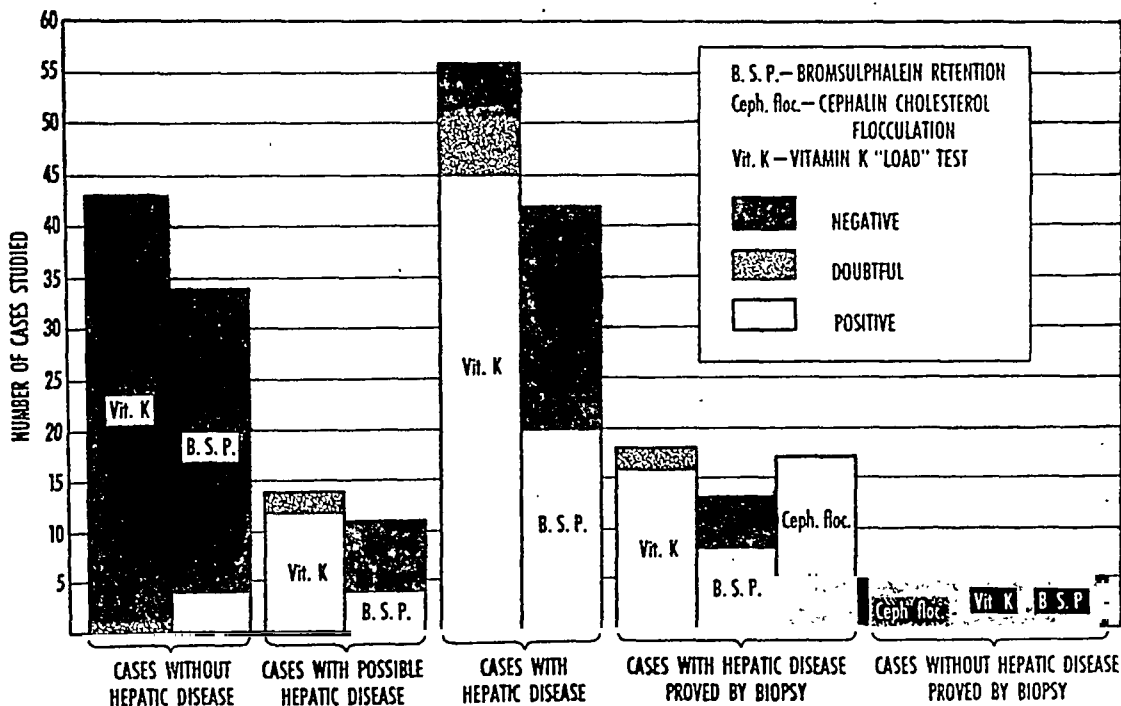


FIG. 3. THE CASES ARE ARRANGED FROM THE VIEWPOINT OF PRESENCE OR ABSENCE OF LIVER DISEASE

In 43 cases without liver disease, none yielded a positive result in the vitamin K tolerance test; in 56 cases with hepatic disease only 5 showed a negative result, and in 18 of these cases in which biopsy studies established the presence of hepatic disease, no negative results were obtained. The results of the vitamin K tolerance tests are correlated with bromsulphalein retention and cephalin cholesterol flocculation.

ful in 1. In 34 of these cases where BSP retention was tested, 30 showed negative and 4 positive BSP tests. There were 14 other cases without evidence of any specific liver disease. Thirteen of these had conditions known to be associated with impaired liver function and 1 subject had no demonstrable disease. The vitamin K tolerance test was positive in 12 and doubtful in 2 of these 14 subjects. BSP retention was tested in 11 of these cases with 4 positive and 7 negative results. There were 56 cases with clinical evidence of liver disease, 45 of which showed positive, 6 doubtful and 5 negative vitamin K tolerance tests. BSP retention was tested in 42 of these cases with 20 positive and 22 negative results. Of the 5 cases with clinical evidence of liver disease and negative vitamin K tolerance tests, 3 had diseases which do not commonly result in impaired liver function (amyloidosis and small hepatic metastases), 1 with a fatty liver which may be associated with impaired liver function, and the remaining case with a disease (cardiac cirrhosis) which may or may not affect liver function. BSP retention was tested

in all 5 of these cases with 4 negative and 1 positive result. From the foregoing analysis of this group of cases it appears that the vitamin K tolerance test exhibits excellent correlation with other clinical findings indicative of impaired liver function referable to various causes.

Analysis of cases with postmortem, biopsy, or operative findings

It was possible also to correlate the results of liver function tests, including the vitamin K tolerance test, with biopsy, postmortem, or operative findings in 24 cases (Figure 3).

In summary, 18 of the 24 cases yielded evidence from tissue study of liver disease. Sixteen of these cases showed a positive and 2 a doubtful vitamin K tolerance test. Thirteen of these 18 cases in which bromsulphalein retention was tested yielded 8 positive and 5 negative results. Four of the 24 patients in whom examination of liver tissue was possible showed no histological evidence of liver disease. In all 4, vitamin K tolerance and BSP tests were both negative. In the remaining

2 cases, 1 of amyloidosis and 1 of cardiac cirrhosis, vitamin K tolerance and BSP tests were both negative. One case, of Simmonds' disease, is worthy of special comment because of the bizarre nature of the response of the prothrombin level to the parenteral administration of vitamin K (Synkayvite). This patient started with a prolonged resting prothrombin time which returned slowly over a course of 10 days to normal values.

The cephalin-cholesterol flocculation test was performed on 22 patients. Among these there were 17 who showed pathological evidence of liver disease. In 5 the cephalin-cholesterol flocculation test was less than 2 plus, and in 12 it was 2 plus or over. In the 5 cases who exhibited both negative vitamin K tolerance and BSP tests, there were 3 cases which showed no pathological evidence of liver disease, but yielded a 2 plus cephalin-cholesterol flocculation test. In 1 case with cardiac cirrhosis, and 1 with amyloidosis, the cephalin-cholesterol flocculation test was negative.

From this review of cases where pathological examination of the liver was obtained, the vitamin K tolerance test appears to be a reliable indicator of hepatic disease and impaired hepatic function.

DISCUSSION

Test of hepatic function as an aid in the diagnosis and prognosis of liver disease is perhaps best served by the use of a number of liver function tests. This is inherent in the fact that the liver serves many functions, has great reserve and regenerative ability (16 to 20). Neefe (19, 20) states that the thymol turbidity, cephalin flocculation, and the colloidal gold tests are the most sensitive indicators of persistent hepatic dysfunction in cases of infectious hepatitis. Unfortunately, plasma prothrombin levels after vitamin K administration were not done. Since the thymol turbidity and cephalin flocculation tests depend upon the presence of beta and gamma globulins, respectively (21), serum protein changes without hepatic cellular involvement may give rise to positive tests.

Neefe and Rheingold (20) found in infectious hepatitis that bromsulphalein retention and bilirubinuria were usually the first tests to indicate early preicteric hepatitis. The results of the bromsulphalein test are believed to depend upon involvement of the polygonal cells of the liver. This test

appears to be of value in the diagnosis of many forms of liver disease. Several factors, however, tend to diminish the reliability of this test. First, bromsulphalein is excreted through the bile passages; hence any lesions, extrinsic or intrinsic, producing biliary tract obstruction will delay the excretion of the dye from the blood stream whether or not it is associated with impaired liver function. Secondly, in conditions of circulatory stasis where complete mixing of injected dye occurs slowly, BSP retention may be abnormal due to circulatory factors rather than hepatic dysfunction. Thirdly, bromsulphalein is a substance foreign to the metabolism of the body. The testing of a normal physiological function would appear to be more desirable.

In the evaluation of any liver function test, its specificity for measuring a particular function is often as important as its reliability and sensitivity. On the basis of the material studied and the analysis of results, the vitamin K tolerance test appears to be a sensitive index of the ability of the liver to produce prothrombin. The results suggest, further, that this function moves parallel with, and is one of the more delicate indicators of, the general functional state of the liver. In certain instances it is more sensitive than the bromsulphalein retention test. The test has also the distinct advantage that it measures a function which apparently resides only in liver tissue. Prothrombin production is also disturbed in vitamin K (nutritional) deficiency but these cases respond readily to the parenteral administration of vitamin K by return of low plasma prothrombin levels to normal. There may be a shift in other components concerned in the clotting mechanism at the same time that prothrombin production becomes reduced. As far as fibrinogen is concerned (22) preliminary studies (23) indicate that it does not play a role in the changes in prothrombin time elicited by the single-stage technique as employed in the present study in liver disease. Further investigation of the problem is in progress in this laboratory and will be reported.

The further depression of the prothrombin level, or activity following the parenteral administration of large doses of vitamin K, suggests that in the cases of hepatic disease studied, the liver appears to be functioning at its maximum capacity under the prevailing conditions. The demonstrated

striking sensitivity of the mechanism suggests that the hepatic reserve for this function is less than that of other known and measurable liver functions. The prolongation of prothrombin time observed after the administration of large doses of vitamin K in liver disease may occur either because of inadequate production of the substrate with which vitamin K combines, or the utilization of this substrate in an abnormal manner and consequent interference with the production of prothrombin. This would reduce the substrate available for prothrombin elaboration. As soon as the substrate is replenished the resting level of prothrombin becomes reestablished.

If the liver stores vitamin K, it seems logical to assume that in hepatic disease this capacity becomes reduced. The normal liver would use its vitamin K stores as needed, restore its supply as needed and thus maintain the prothrombin activity at a constant level.

Whatever the mechanism, the sudden influx of a large quantity of vitamin K may tend to accelerate prothrombin production. This is well demonstrated in some normal subjects in which a temporary hyperprothrombinemia occurs following the injection of vitamin K derivatives in large doses (24). The response of a diseased liver to such stimulation might be reflected in an exhaustion phenomenon with consequent prolongation of the prothrombin time. It is noteworthy that this reaction had been observed in only a certain proportion of persons examined. Some retain a fixed level of prothrombin despite administration of massive doses of vitamin K. This is true of normals as well as cases of liver disease.

In all cases in which existing hypoprothrombinemia was exaggerated by large doses of vitamin K, the phenomenon was transitory, lasting only 24 to 48 hours. Nevertheless, it is suggested that in patients with hepatic disease in whom the prothrombin time is already prolonged, that if vitamin K is used, a small dose be given with repetition only where the prothrombin time does not further increase. It is particularly important where hemorrhage has occurred recently, or where the tendency to bleed is present. The only manner in which prothrombin activity can be augmented in such cases is by means of transfusion, preferably of whole, fresh blood, or possibly by administration

of frozen plasma (6). None of the cases in our series exhibited bleeding phenomena.

Although our results indicate that the vitamin K tolerance test shows a high degree of sensitivity for detecting slight degrees of hepatic functional impairment, the test cannot serve alone to establish the presence or absence of liver disease. It should be correlated with all other means of clinical examination and appropriate function tests to form an inventory, so to speak, of the state of the liver. The value of biopsy study, particularly in doubtful cases, cannot be overestimated as confirmatory evidence.

It is not yet clear that a particular type of response is of prognostic value. Repeated tests in the same patient, in a large series of cases over an extended period of time, and after different types of therapy, may help determine this important point. The simplicity of performance of the vitamin K tolerance test permits its application on a large scale.

SUMMARY AND CONCLUSIONS

1. A standardized vitamin K tolerance test estimating the prothrombin response to the parenteral administration of large test doses of vitamin K has been used.

2. In a series of 57 cases without clinical evidence of liver disease, and 56 cases with clinical evidence of liver disease, the results have been correlated with other liver function tests. Twenty-four of the 113 cases had morphologic study of the liver.

3. The vitamin K tolerance test exhibits excellent correlation with other clinical findings indicative of impaired liver function referable to various causes.

4. The vitamin K tolerance test was found to be a sensitive indicator of hepatic function.

5. This test is one of considerable sensitivity for measuring the hepatic function of prothrombin formation and can serve as a good index of the presence or absence of hepatic disease.

The thromboplastin used in these studies was supplied by the Maltine Co., Brooklyn, N. Y.

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THE RENAL REGULATION OF ACID-BASE BALANCE IN MAN.

I. THE NATURE OF THE MECHANISM FOR ACIDIFYING THE URINE¹

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Under normal conditions the urine is more acid in reaction than the plasma from which it is formed, and some 100 to 300 ml. of one-tenth normal alkali are required to titrate the 24-hour specimen to pH 7.4 (1). When the metabolic acid load on the body is increased in diabetic ketosis, as much as 1200 ml. of one-tenth normal titratable acid may be excreted per day (2). Since the excretion of acid in free titratable form makes available to the body an equivalent quantity of fixed base for conversion into bicarbonate, the renal mechanisms involved in acidifying the urine play a significant role in stabilizing the alkali reserve (3).

There are obviously 2 ways in which acid might gain access to the urine. First, it might be present as free buffer acid in the glomerular filtrate. If during passage of the filtrate through the renal tubules, alkaline buffer components were preferentially reabsorbed, the reaction of the urine would shift toward the acid side. Second, the acid might be added to the filtrate by some active transport mechanism resident in the renal tubular cells. The 4 possible permutations of these 2 theories are presented in diagrammatic form in Figure 1.

Only 2 acids exist in the plasma in significant quantities in freely filtrable form: monobasic phosphate and carbonic acid. Each, associated with its alkaline counterpart, dibasic phosphate and bicarbonate, passes into the glomerular filtrate. If dibasic phosphate were reabsorbed by the renal tubules, the excreted monobasic phosphate could be titrated as acid in the urine (1). On the other hand, if bicarbonate were reabsorbed from the filtrate, and if the renal tubules were relatively impermeable to carbonic acid (in reality dissolved

carbon dioxide), this acid could react with buffer salts and convert them into free titratable acid (4).

Similarly 2 hypothetical mechanisms depending on secretory activities of the tubular epithelium have been proposed as explanations of urinary acidification. The tubular secretion of molecular acid has been invoked to account for the conversion of the alkaline buffer components of the glomerular filtrate into their acid forms (5). On the other hand a pseudosecretory or ionic-exchange mechanism accomplishing the same end has been suggested (6). According to this latter view, hydrogen ions, formed within the tubular cells by the dissociation of carbonic acid, are exchanged for ions of fixed base bound by buffer salts in the tubular urine, thereby converting them into titratable buffer acids.

Recent studies on the dog (7, 8, 9) have indicated that the induction of acidosis and the infusion of suitable buffers greatly facilitate the excretion of titratable acid. By measuring simultaneously the rate of filtration and reabsorption of phosphate and the rate of filtration of carbonic acid, it was demonstrated that the phosphate and carbonic acid filtration-reabsorption concepts are inadequate to explain the origin of urinary acid. Thus, by exclusion, it was shown that the tubular cells must add acid to the urine. Indirect evidence favored ionic exchange as the more reasonable of the 2 alternative mechanisms.

The present study of the mechanism of acidification of the urine by the normal human kidney was undertaken with 2 purposes in mind: first, to identify the nature of the mechanism; and second, to compare the acid excretory capacity of the human kidney with that of the dog. It has been found that the mechanisms are qualitatively similar in man and dog. Quantitatively, the capacity

¹ Aided by grants from the United States Public Health Service and the John and Mary R. Markle Foundation.

of the normal human kidney to excrete acid appears to exceed that of the dog.

METHODS

The 8 experiments forming the basis of this report, were performed on 4 healthy adult male subjects. Acidosis was induced by the ingestion of 20 grams of ammonium chloride on the day preceding the experiment. The salt was divided into 10 equal doses and was taken well diluted with water at hourly intervals throughout the day. No food was taken following the evening meal. Two glasses of water were ingested the morning of the experiment. Urines were collected by catheter and the bladder was washed with distilled water at the end of each collection period. Blood was drawn from 1 of the superficial veins of the forearm after soaking the limb in water at 47° to 48° C. for 5 to 7 minutes to render the composition of the venous blood essentially the same as that of arterial blood. For measurement of pH and carbon dioxide content, the blood was handled anaerobically (10). Analytical procedures for thiosulfate, phosphate, creatinine and urinary titratable acid and ammonia have been described in previous communications (7, 11).

Glomerular filtration rate was assessed by the thiosulfate clearance (12). Plasma thiosulfate concentrations were maintained between 30 and 40 mgm. per cent by continuous intravenous infusions. Both urine and plasma blanks were determined and appropriate corrections applied in all calculations. Neutral sodium phosphate² (pH 7.4) was incorporated in the infusions and volume was adjusted by the addition of distilled water to render all solutions isotonic. The rate of infusion was varied between 3 and 13 ml. per minute to attain the desired plasma concentrations. No adverse reactions were observed from the infusion of phosphate; nausea and emesis followed the infusion of creatinine. At the highest plasma phosphate levels (6 to 7 mM per liter) minimal signs of tetany were observed which subsided when the infusions were stopped.

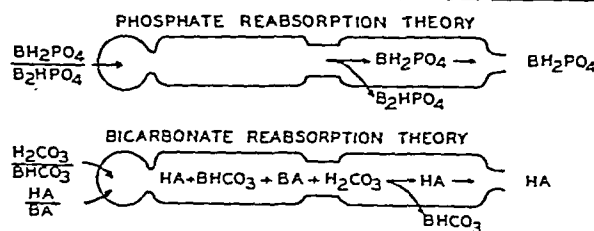
RESULTS

Experiments with phosphate

Our plan of attack in identifying the nature of the renal mechanism for acidifying the urine has been to increase the rate of excretion of titratable acid by the induction of acidosis and by the infusion of large quantities of sodium phosphate. By measuring the quantities of phosphate filtered, reabsorbed and excreted and the quantity of carbonic acid filtered it has been possible to calculate exactly the maximum contribution which the hy-

THEORIES TO ACCOUNT FOR THE EXCRETION OF ACID URINE

URINARY ACID PRESENT IN ORIGINAL FILTRATE



URINARY ACID SECRETED INTO TUBULAR URINE

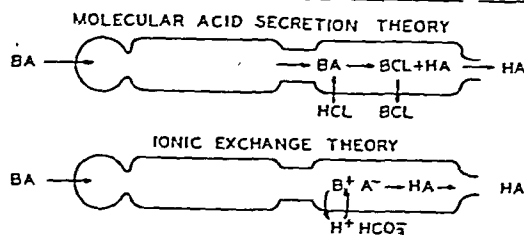


FIG. 1. FOR EXPLANATION, SEE TEXT

pothetical phosphate and carbonic acid filtration-reabsorption mechanisms could possibly make to the excretion of titratable acid. Since monobasic phosphate and carbonic acid are the only acids present in significant amounts in a protein-free ultrafiltrate of plasma, it is obvious that if these substances cannot account for the observed rate of excretion of titratable acid, acid must be added to the urine by one or the other of the 2 active cellular transport mechanisms. Experiments presented below are conclusive in showing that tubular mechanisms are responsible for the addition of acid to the urine under our conditions of stress.

Experiment 1 in Table I illustrates both the character of the measurements and the relative significance of acidosis and plasma phosphate concentration in the experimental procedures employed. A moderately severe yet well compensated acidosis was induced by the ingestion of ammonium chloride. The extent of the acidosis is apparent in the plasma bicarbonate concentration of 14.2 mM per liter, and the degree of compensation is evident both in the plasma pH of 7.37 and in the plasma carbonic acid concentration of 0.77,³ shown in the first 2 periods of Experiment 1. In these

² We are indebted to the William R. Warner Co. of New York for the preparation of a generous supply of ampoules of the sterile pyrogen-free sodium phosphate solution of pH 7.4 used in this work.

³ Normal values for R. F. P. are bicarbonate, 24 to 25 mM per liter; pH, 7.39 to 7.41; carbonic acid, 1.17 to 1.23 mM per liter.

TABLE I

Critical experiments on man designed to test the several theories of urinary phosphate excretion.
The major urinary buffer in these experiments was phosphate.

Subject and experiment number	Glomerular filtration rate	Urine flow	Plasma concentration			Phosphate		Rate of excretion of titratable acid						Ratio of ammonia to titratable acid	Rate of ammonia excretion	Ob- served	Urine pH			
			BHCO ₃	H ₂ CO ₃	pH	Phos- phosphate	Excreted	Reab- sorbed	Observed	Calculated from							Ob- served	Calculated from		
										Phosphate excreted	Carbonic acid filtered		Phosphate reabsorbed							
											per cent of ob- served	mEq./min.	per cent of ob- served						mEq./min.	per cent of ob- served
R. F. P. 1	95.5	4.07	14.2	0.77	7.37	1.10	0.022	0.083	0.030	0.017	56.6	0.074	100+	0.017	56.6	2.03	0.061	4.49	adeq.	
	88.8	3.27				0.92	0.020	0.061	0.027	0.016	59.2	0.068	100+	0.013	48.1	2.22	0.060	4.55	adeq.	
	96.7	3.87	14.6	0.79	7.37	1.73	0.063	0.104	0.062	0.050	80.7	0.076	100+	0.022	35.5	1.05	0.065	4.58	adeq.	
	103	6.81				2.13	0.108	0.111	0.096	0.085	88.6	0.081	84.4	0.023	24.0	0.75	0.072	4.51	6.70	
R. F. P. 2	98.6	14.6	14.0	0.80	7.35	3.70	0.238	0.127	0.182	0.184	101	0.079	43.4	0.028	15.4	0.37	0.068	4.65	6.01	7.10
	107	17.3				4.13	0.324	0.118	0.247	0.251	101	0.086	34.8	0.026	10.5	0.28	0.070	4.64	6.13	7.17
	88.2	9.0	14.9	0.81	7.37	5.78	0.388	0.122	0.293	0.303	103	0.071	24.2	0.025	8.5	0.16	0.046	4.56	6.28	7.21
	88.4	9.1				6.52	0.443	0.133	0.333	0.346	104	0.072	21.6	0.028	8.4	0.17	0.056	4.56	6.32	7.22
W. A. S. 3	102	7.53	14.8	0.86	7.34	5.45	0.419	0.137	0.328	0.322	98.2	0.087	26.5	0.031	9.5	0.20	0.066	4.64	6.92	7.17
	101	8.40	14.8	0.86	7.34	6.04	0.455	0.155	0.348	0.350	100.5	0.087	25.0	0.035	10.0	0.19	0.064	4.63	6.95	7.17
	98.7	8.74	14.6	0.82	7.35	6.49	0.486	0.154	0.371	0.376	101.4	0.081	21.8	0.034	9.2	0.17	0.062	4.60	7.00	7.19
	100	7.73	14.8	0.83	7.35	6.73	0.516	0.157	0.395	0.399	101.1	0.083	21.0	0.035	8.9	0.15	0.061	4.56	7.01	7.19
W. A. S. 4	100	12.9	16.6	0.95	7.34	5.57	0.443	0.114	0.318	0.329	104.0	0.095	29.9	0.026	8.2	0.24	0.076	5.34	6.91	7.21
	109	16.4	16.8	0.96	7.34	5.92	0.517	0.128	0.366	0.380	104.0	0.105	26.2	0.029	7.9	0.21	0.077	5.42	6.93	7.21
	113	18.5	16.7	0.93	7.35	6.58	0.590	0.154	0.410	0.429	105.0	0.105	25.6	0.034	8.3	0.19	0.077	5.54	6.98	7.22
	120	18.5	16.8	0.94	7.35	6.73	0.646	0.162	0.451	0.470	104.0	0.113	25.0	0.036	8.0	0.18	0.080	5.54	6.99	7.22
J. L. A. 4	127	12.8	19.8	1.04	7.38	4.56	0.435	0.144	0.342	0.335	98.0	0.132	39.7	0.030	8.8	0.22	0.077	5.11	6.78	7.21
	131	14.7	19.8	1.04	7.38	4.81	0.475	0.155	0.362	0.364	100.5	0.136	37.6	0.033	9.1	0.20	0.074	5.21	6.81	7.21
	114	13.1	20.1	1.06	7.38	5.01	0.428	0.144	0.324	0.326	100.6	0.121	37.3	0.030	9.3	0.19	0.063	5.28	6.82	7.21
	123	14.4	20.5	1.03	7.40	5.37	0.498	0.162	0.374	0.382	102.1	0.127	34.0	0.033	8.8	0.18	0.068	5.31	6.87	7.21
W. D. L. 5	138	14.8	19.8	0.95	7.42	4.00	0.379	0.173	0.290	0.304	104.8	0.131	45.2	0.034	11.7	0.21	0.061	4.50	6.73	7.20
	148	16.7	19.8	0.95	7.42	4.26	0.449	0.181	0.354	0.360	102.0	0.141	39.8	0.035	9.9	0.18	0.065	4.50	6.78	7.23
	151	15.5	19.9	0.95	7.42	4.26	0.463	0.180	0.368	0.371	100.8	0.144	39.1	0.035	9.5	0.18	0.066	4.48	6.79	7.23
	150	15.1	20.0	1.00	7.41	4.67	0.495	0.205	0.388	0.394	101.5	0.150	38.7	0.040	10.3	0.16	0.063	4.60	6.80	7.21

2 control periods the concentration of phosphate in the plasma ranged between 1.10 and 0.92 mM per liter, or 0.00110 and 0.00092 mM per milliliter. If these latter figures are multiplied by the glomerular filtration rate, one obtains the quantity of phosphate filtered per minute. The product of the urine concentration and urine flow yields the quantity of phosphate excreted per minute. The quantity of phosphate reabsorbed, equal to the difference between the quantities filtered and excreted, amounted initially to 0.083 and 0.061 mM per minute. Assuming this reabsorbed phosphate to be the dibasic form, the residual excess of monobasic phosphate left in the urine could account for the excretion of 0.017 and 0.016 mEq of titratable acid per minute, or 56.6 to 48.1 per cent of that actually observed. Thus, acidosis alone rendered the phosphate reabsorption concept untenable in this experiment. In contrast, the filtration of carbonic acid is capable of explaining 100 per cent of the titratable acid eliminated in the 2 control periods.

In the succeeding 6 periods the plasma phosphate concentration was elevated in stepwise fashion to a maximum of 6.52 mM per liter by the infusion of neutral sodium phosphate. The reabsorption of phosphate reached a maximum of 0.120 to 0.130 mM per minute and the excess filtered, over and above this quantity reabsorbed, was excreted in the urine. The observed rate of excretion of titratable acid increased in linear fashion with the increased rate of excretion of phosphate despite the relatively constant rate of reabsorption. Accordingly, the phosphate reabsorption concept became progressively a less adequate explanation of urinary acidity, until in the final periods it could account for only 8 per cent of the observed acid. The quantity of carbonic acid filtered remained essentially constant throughout the experiment, varying only between 0.068 and 0.086 mEq per minute. Accordingly from the fourth period on, the carbonic acid filtration concept likewise became an inadequate explanation of urinary acidity and in the final period could account for only 21.6 per cent of the acid excreted. It is apparent that titratable acid excretion can be increased to levels greatly above normal by increasing the quantity of phosphate excreted. In fact in the eighth period of Experiment 1, a rate of excretion of 0.333 mEq per min-

ute is equivalent to the elimination of 4750 ml. of one-tenth normal acid per day. This is some 3 to 4 times the highest ever observed in diabetic ketosis. At plasma phosphate concentrations of 6 to 7 mM per liter, the 2 hypothetical filtration-reabsorption mechanisms together can account for only one-third of the titratable acid excreted. Since these are the only significant sources of acid in the filtrate it is apparent that acid must be added to the tubular urine by some active cellular transport mechanism. It seems reasonable to us to assume that this cellular mechanism accounts for acid excretion under normal conditions as well as under conditions of acidosis and hyperphosphatemia, although it is impossible to quantify its activity except by exceeding the limited acid excretory capacity of the 2 filtration-reabsorption mechanisms. We likewise feel from theoretical considerations discussed at length in a previous paper (7), that under no circumstances do phosphate reabsorption and carbonic acid filtration contribute significantly to the titratable acid of the urine.

With the information derived from this preliminary experiment, identical 4-period experiments were performed on 4 subjects, the results of which are presented in Table I. The extent of the acidosis varied considerably in the several subjects, as did the plasma concentration of phosphate. Variations in filtration rate, phosphate reabsorptive capacity and plasma carbonic acid concentration combined to alter the relative adequacies of the phosphate reabsorption concept and the carbonic acid filtration concept as explanations of the observed rates of excretion of titratable acid. In these latter 4 experiments phosphate reabsorption could account for only 8.0 to 11.7 per cent of the excreted acid. Carbonic acid filtration could account for only 21 to 40 per cent of the excreted acid. Both mechanisms together could account for only one-third to one-half of the acid. Thus, it is apparent that acid must be added to the urine by the renal tubules.

The columns in the center of the table headed *rate of excretion of titratable acid calculated from phosphate excreted* serve as a check on the accuracy of the chemical determinations, at least in those instances in which the rate of excretion of phosphate was high. In Experiments 2 to 5, values for titratable acid calculated from the pH

of the urine and the rate of excretion of phosphate should agree with the observed values within reasonably small limits. The calculated values average 101.8⁴ per cent of the observed values. Actually the calculated values should average slightly less than 100 per cent, for there is a small moiety of non-phosphate titratable acid in the urine made up of creatinine and organic acid. However, the agreement is sufficiently close to warrant confidence in conclusions drawn from the more rigorous analysis of the data. In the initial 2 periods of Experiment 1, phosphate constituted between 50 and 60 per cent of the titratable acid; about half of the remainder was creatinine, about half was organic acid. As the excretion of phosphate and titratable acid increased, this nonphosphate moiety became a less and less significant proportion of the total. Whether it remained constant or diminished cannot be stated, because the range of experimental error of the determination at high phosphate levels is greater than this moiety.

The rate of ammonia excretion was followed in each experiment and averaged 0.066 mEq per minute in the series presented in Table I. Although considerably increased over the normal, this rate of excretion is not high in comparison with that observed in diabetic ketosis, no doubt because the acidosis was not especially severe and was of short duration (less than 24 hours). In normal individuals and in patients in diabetic ketosis as well, the ratio of ammonia to titratable acid in the urine varies within limits of 1.0 and 2.5 (13). In the initial 2 periods of Experiment 1 the ratio amounted to 2.03 and 2.22, well within the usual limits. Following the infusion of phosphate the ratio dropped progressively to a low of 0.16. This drop was brought about for the most part by the increase in rate of titratable acid excretion, not by diminution in ammonia elimination. Thus, the magnitude of this ratio in the presence of normal renal function is conditioned largely by the quantity and properties of the urinary buffers.

It is obviously possible to analyze the data in another way, namely in terms of the pH of the urine. The pH of the urine was calculated from

⁴ The pK' for phosphate has been taken to be 6.80. Obviously variation in this value in different urine samples is a possible source of error.

the measured rate of buffer excretion assuming first that the only source of acid is filtered carbonic acid, and second that the source of acid is the excess monobasic phosphate remaining in the tubules following reabsorption of dibasic phosphate. The results of this analysis are presented in the last 3 columns of Table I. In order to perform this calculation it has been necessary to assume a pK' for the non-phosphate moiety of the titratable acid. This has been taken to be that of creatinine, namely 4.97, an assumption which is only partially justified. Except in the initial 2 periods of Experiment 1, no serious error is introduced in the calculations if this assumed pK' is erroneous. It is apparent from Experiment 1 that the phosphate reabsorption concept and the carbonic acid filtration concept are adequate to explain the pH of the urine only when the rate of buffer excretion is low. As the buffer content of the urine is increased by the infusion of phosphate, the deviations between observed and calculated urine pH values become large. It is interesting that 2 subjects, R. F. P. and W. D. L., were able to excrete urines containing large quantities of phosphate, yet of an acidity considerably greater than that assumed to be possible by the human kidney. The generally accepted limit of acidity of human urine is pH 4.7 (14). Actually in 3 experiments on these 2 subjects all urines ranged in acidity between pH 4.48 and 4.60.

Experiments with creatinine

In order to test further the thesis that the urine is acidified by tubular secretory processes, we repeated the experiments substituting creatinine for phosphate as the buffer administered. Three experiments were performed on 3 different subjects. In the first experiment neither the quantity of creatinine administered nor the degree of acidosis was sufficiently great to yield conclusive results. The 2 more significant experiments are presented in Table II.

In Experiment 7 the acidosis was moderate; in Experiment 8, somewhat more severe; in both, the acidosis was well compensated. Sufficient creatinine was administered to raise the plasma concentration to 20 to 33 mgm. per cent and to effect the excretion of 0.296 to 0.466 mM of this buffer per minute. The rate of elimination of endogenous phosphate varied between 0.016 and

TABLE II
Critical experiments on man designed to test the several theories of urinary acidification
 The major urinary buffer in these experiments was creatinine

Subject and experiment number	Plasma concentration					Excreted		Rate of excretion of titratable acid								Urine pH		
	Glo- merular filla- tion rate	Urine flow	H ₂ CO ₃	pH	Crea- tine	Phos- phate	Crea- tine mM/ min.	Phos- phate mM/ min.	Ob- served	Calculated from						Ob- served	Calculated from	
										Total buffer excreted	Carbonic acid filtered	Phosphate reabsorbed	per cent of ob- served	mEq/ min.	per cent of ob- served		Car- bonic acid filtered	Phos- phate reab- sorbed
W. A. S. 7	107	3.26	17.3	7.38	31.8	0.88	0.466	0.025	0.173	0.147	0.019	0.015	96.0	0.097	56.0	5.31	5.67	6.37
	97.8	2.93	17.2	7.38	33.3	0.80	0.433	0.021	0.168	0.145	0.016	0.012	95.8	0.088	52.4	5.27	5.67	6.42
	100	3.00	16.9	7.38	33.9	0.76	0.445	0.019	0.179	0.153	0.015	0.012	93.8	0.089	49.7	5.25	5.67	6.40
	98.1	2.73	16.8	7.38	33.9	0.72	0.429	0.017	0.175	0.157	0.013	0.011	97.2	0.086	49.1	5.21	5.67	6.44
R. F. P. 8	102	3.33	12.5	7.38	22.3	0.73	0.329	0.026	0.167	0.149	0.021	0.010	101.8	0.067	40.1	5.05	5.76	6.31
	103	3.07	12.5	7.38	20.3	0.70	0.296	0.022	0.169	0.146	0.017	0.010	103.6	0.068	40.2	4.98	5.64	6.27
	107	3.07	13.0	7.39	31.9	0.67	0.456	0.014	0.203	0.194	0.011	0.011	101.0	0.072	35.4	5.10	5.78	6.47
	110	3.07	13.0	7.39	29.8	0.66	0.435	0.016	0.203	0.187	0.013	0.011	98.7	0.074	36.4	5.07	5.76	6.45

0.026 mM per minute. The values for titratable acid calculated from the urine pH and the sum of the creatinine and phosphate eliminated varied from 0.161 to 0.205 mEq per minute. The observed rates of titratable acid excretion varied from 0.167 to 0.203 mEq per minute, values much lower than those observed following phosphate administration. Nevertheless, calculated and observed values for titratable acid agreed within reasonable limits.

It is evident from the calculations of the maximum possible contributions of phosphate reabsorption and carbonic acid filtration to titratable acid excretion that neither concept adequately explains acidification of the urine. Thus, carbonic acid filtration could account for only 35 to 56 per cent, and phosphate reabsorption for only 5.4 to 8.7 per cent of the excreted acid. Similarly an analysis of the possible contributions of the 2 mechanisms to the excretion of urine of low pH, indicates their essential incapacity to account for the degree of acidity actually obtained. Although the deficiencies of the 2 hypothetical filtration-reabsorption mechanisms are somewhat less striking in these experiments with creatinine than in the phosphate ones, they do confirm the thesis that active tubular processes must add acid to the urine. This confirmation is especially significant in view of the differences in the mechanisms by which the human kidney handles phosphate and creatinine: the former by filtration and reabsorption, the latter by filtration and secretion (15).

DISCUSSION

The data presented above are conclusive in demonstrating that under our experimental conditions the normal human kidney can excrete more acid per unit of time than could gain access to the urine in the glomerular filtrate. Therefore, by exclusion, acid must be added to the urine by some mechanism of active tubular transport. As pointed out above, this mechanism might be one of tubular secretion of molecular acid, or one of exchange of hydrogen ions formed within the renal tubular cells for ions of fixed base in the tubular urine. Our experimental methods cannot distinguish between these 2 possibilities. Indeed, as pointed out in previous work on the dog (7) the question is largely an academic one. If molecular acid,

e.g., hydrochloric acid, were secreted by the renal tubules, the sodium chloride formed in its reaction with buffer salts would be largely reabsorbed further along the tubules. Therefore, in effect, an exchange of hydrogen ions for sodium ions would be brought about by secretion of molecular acid followed by reabsorption of neutral salt. Because the various salts in plasma, glomerular filtrate and urine are dissociated, it seems reasonable to assume that the kidney operates on ions rather than on molecular species, within the limitations imposed by the necessity for maintaining the electroneutrality of these several fluids. An ionic exchange mechanism would accomplish in 1 step the conservation of base and the excretion of acid, and incidentally satisfy requirements of electroneutrality.

Our experimental results on normal human subjects are so similar to those previously published on dogs that it seems legitimate to assume that the renal mechanisms are identical. One may therefore be permitted to extrapolate from certain experiments performed on dogs, too rigorous to be applicable to man, to fill in gaps in knowledge of the human renal mechanism. Obviously carbonic acid, formed by the hydration of carbon dioxide, is the only source of hydrogen ions available to the body in quantities sufficient to satisfy the demands imposed by the observed rates of urinary acid excretion. Kidney tissue contains large amounts of carbonic anhydrase, an enzyme which markedly increases the rate of hydration of carbon dioxide to carbonic acid (16). Sulfanilamide, an inhibitor of carbonic anhydrase (17), when present in the plasma in high concentration (30 to 80 mgm. per cent) greatly reduces or abolishes the excretion of titratable acid in the dog (7). From these facts it was concluded that carbonic anhydrase is an essential link in the tubular ionic exchange mechanism, and that its role is to speed the hydration of carbon dioxide to carbonic acid within the cells of the renal tubules. The dissociation of this carbonic acid provides hydrogen ions for exchange with base bound by buffers of the tubular urine. The base in association with bicarbonate ions is then returned to the renal venous blood. We feel that these conclusions regarding the nature of the tubular mechanism in so far as they are valid for the dog, are

equally applicable in man, especially in view of the disturbances in acid-base balance described in the early clinical use of sulfanilamide (18). Indeed we feel that one of the most significant aspects of the present investigation lies in its justification of the use of the dog for analysis of problems of acid-base regulation.

In a quantitative sense, we feel that the capacity of the human kidney to excrete acid probably exceeds that of the dog. This statement may not be generally valid for it is based only upon a comparison of 4 healthy young adults with 4 healthy mongrel dogs. But the fact that the results are so consistent seems to justify this tentative statement. In experiments on dogs (7) similar to those reported above, the highest rate of titratable acid excretion which was observed amounted to 0.380 mEq per minute. This was attained at a plasma phosphate level of 9.84 mM per liter and at a rate of excretion of phosphate of 0.613 mM per minute. In attaining this rate of acid excretion, the kidney elaborated a urine of pH 6.07; in essence the kidney could establish a concentration gradient for hydrogen ions between tubular urine and plasma of somewhat less than 20 to 1. The 4 human subjects excreted from 0.374 to 0.451 mEq of titratable acid at plasma phosphate concentrations which were substantially lower than those of the dogs. Furthermore, at comparable rates of phosphate excretion, urines of much lower pH (4.48 to 5.54) were elaborated by the human subjects. Thus the human kidney can transfer the same or larger quantities of hydrogen ions against higher concentration gradients. Subjects W. A. S. and J. L. A. could establish a gradient of roughly 100 to 1 while transferring approximately 0.400 mEq of hydrogen ions per minute; subjects R. F. P. and W. D. L. could establish a gradient of 800 to 1 while transferring equivalent quantities of hydrogen ions. The difficulties of comparing acid excretory capacity in dog and man not only lie in differences in body and kidney size, but likewise in the fact that in neither dog nor man was any absolute maximum for acid excretion attained. Doubtless further increases in the rate of phosphate excretion in both forms would have led to further increases in acid excretion.

SUMMARY

In a series of 8 experiments on 4 healthy human subjects it was found that the induction of acidosis by the ingestion of ammonium chloride and the promotion of buffer excretion by the infusion of phosphate or creatinine greatly increased the rate of excretion of titratable acid. Simultaneous measurement of the rate of filtration, reabsorption and excretion of mono- and dibasic phosphate and carbonic acid demonstrated that the quantity of acid excreted far exceeded that which entered the urine in the glomerular filtrate. Therefore, acid must have been added to the filtrate as it passed along the nephron by some mechanism of active transport resident in the renal tubular cells. It is suggested that this addition of acid is effected by the exchange of hydrogen ions formed within the tubular cells for ions of fixed base in the tubular urine. Carbonic acid is doubtless the intracellular source of hydrogen ions.

This mechanism for acid excretion in the human kidney is qualitatively similar to that previously described in the dog. Quantitatively, the human kidney has a greater acid excretory capacity than the dog kidney.

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THE RENAL REGULATION OF ACID-BASE BALANCE IN MAN.

II. FACTORS AFFECTING THE EXCRETION OF TITRATABLE ACID BY THE NORMAL HUMAN SUBJECT¹

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Transformation of the slightly alkaline glomerular filtrate into acid urine has been assigned in man (1) as in the dog (2) to the exchange of hydrogen ions formed within the tubular cells for ions of fixed base in the tubular urine. The most acid urine which the kidney can form is of pH 4.5 to 4.7 (1, 3). At this reaction negligible quantities of strong acids such as hydrochloric or sulfuric can exist in free form. Accordingly the exchange of hydrogen ions for base bound by chloride and sulfate in the tubular urine is quantitatively insignificant. But weak buffer acids such as monobasic phosphate, uric acid, creatinine, and beta-hydroxybutyric and aceto-acetic acids can exist in considerable proportion in free titratable form in urine of such acidity (4). As a consequence, the exchange of hydrogen ions for base bound by weak buffer acids in the tubular urine plays a significant role in stabilizing the alkali reserve of the body (5).

It has been demonstrated in the dog that the major determinants of the rate of excretion of titratable acid are (1) the rate of excretion of buffer; (2) the acid strength of the buffer excreted; and (3) the degree of acidosis, as reflected in the bicarbonate content of the plasma (6). Each of these factors appears to play a role in clinical acidosis in man. Thus, in severe diabetic ketosis, a high rate of excretion of titratable acid is associated with a low alkali reserve and with a high rate of excretion of ketone bodies (4). The ketone bodies are relatively strong buffer acids. Accordingly the kidney can salvage from them only a portion of the base they bind in the plasma and glomerular filtrate, and although the rate of titratable acid excretion is high in diabetic

ketosis, a considerable complement of base is lost in the urine.

In the present study the role played by these several factors mentioned above has been investigated systematically in normal human subjects in whom acidosis has been induced by the ingestion of ammonium chloride. By infusing phosphate, creatinine and para-aminohippurate at progressively increasing rates to cause the elimination in the urine of increasing quantities of these buffers, it has been found in man as in the dog that (1) the rate of excretion of titratable acid increases in proportion to the rate of excretion of buffer; (2) at any given molar rate of excretion of buffer the quantity of titratable acid eliminated per unit of time is greatest for the buffer of lowest acid strength (phosphate) and least for the buffer of greatest acid strength (para-aminohippurate); and (3) the rate of excretion of titratable acid is, within limits, proportional to the degree of reduction of bicarbonate in the plasma.

METHODS

The 12 experiments forming the basis of this report were performed on 3 normal adult male subjects. However, the data presented in this paper have been restricted to those obtained on a single subject on whom a complete set of experiments was performed. Additional experiments on the other 2 subjects confirmed the observations recorded here. Experimental methods employed were reported in the preceding paper (1).

RESULTS

The relationship between the rate of excretion of titratable acid and the rate of excretion of buffer

It is generally recognized that the majority of the titratable acid of normal human urine is monobasic phosphate, and that changes in the rate of excretion of endogenous phosphate are accom-

¹ Aided by grants from the United States Public Health Service and the John and Mary R. Markle Foundation.

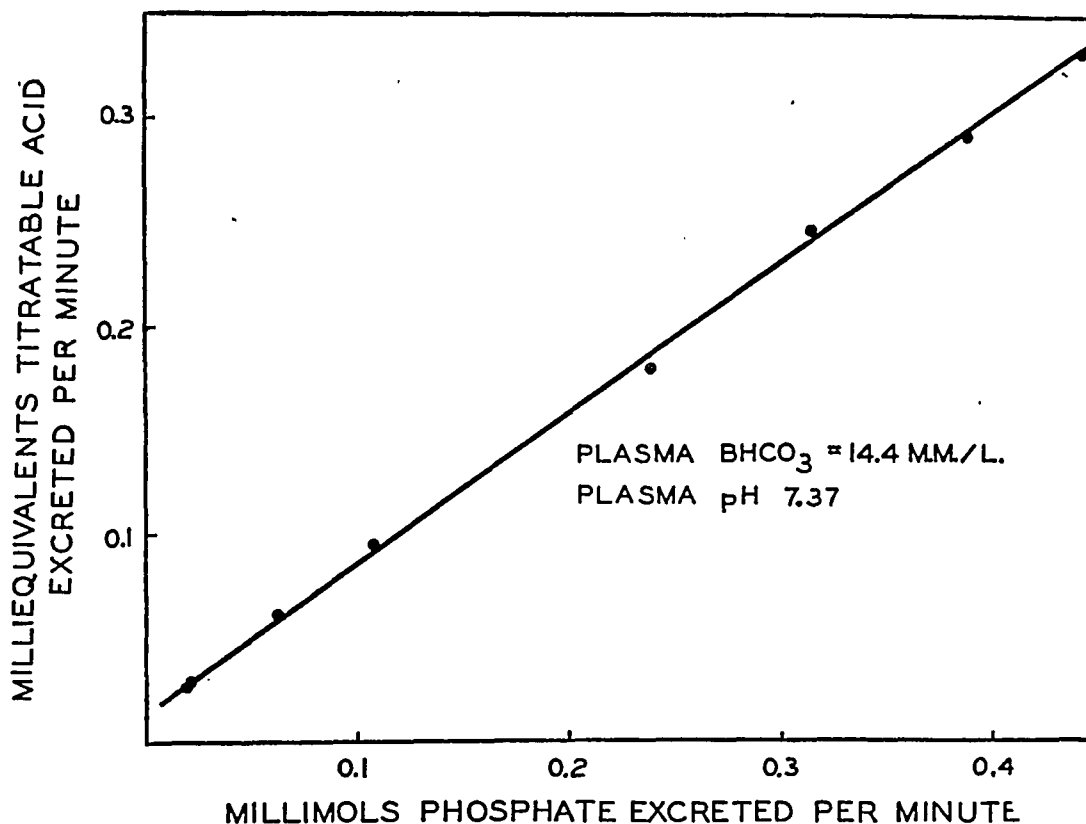


FIG. 1. THE RELATION BETWEEN THE RATE OF EXCRETION OF PHOSPHATE AND THE RATE OF EXCRETION OF TITRATABLE ACID IN MAN IN ACIDOSIS

Data from an experiment on subject R. F. P.

panied by equivalent changes in the rate of excretion of titratable acid (5). In Figure 1 are presented the results of a systematic study of the relationship between urinary phosphate and titratable acid, carried out over a far wider range of phosphate excretion than ever occurs spontaneously. The data are derived from Experiment 1 in Table I of the preceding paper (1). The subject was in a state of moderate acidosis characterized by a plasma bicarbonate concentration of 14.4 mM per liter and a plasma pH of 7.37. The first 2 points in the lower left-hand corner of this figure represent control rates of excretion of titratable acid prior to the administration of phosphate. About 50 per cent of the acid eliminated in these 2 periods can be accounted for as monobasic phosphate derived from endogenous stores. The remainder is creatinine and other organic acids. Neutral sodium phosphate (pH 7.4)² was then infused at progressively increasing rates to increase

the rate of phosphate excretion to a maximum of 0.443 mM per minute. The rate of excretion of titratable acid increased in direct proportion to the rate of excretion of phosphate. Throughout the entire series of observations the urine reaction varied only between pH 4.49 and 4.65. Thus phosphate, which entered the glomerular filtrate mainly in the dibasic form, was eliminated in the urine entirely in the monobasic form. Indeed at such acidities traces of free phosphoric acid are excreted. These results in man differ slightly from those observed in the dog, in which a curvilinear relationship was noted between the rates of excretion of titratable acid and phosphate (6). Deviation from a linear relationship indicates that the kidney of the dog is unable to elaborate as acid a urine at high rates of phosphate excretion as at low rates. This fact is in accord with the statement in the preceding paper (1), that the renal tubular capacity of the dog to acidify the urine is less than that of man. The proportionality between the rate of excretion of acid and the rate of excretion of buffer which is evident in Figure 1 for phosphate, is equally true for other buffers (*cf.* Figure 2).

² We are indebted to the William R. Warner Co. of New York for the preparation of a generous supply of ampoules of the sterile pyrogen-free sodium phosphate solution of pH 7.4 used in this work.

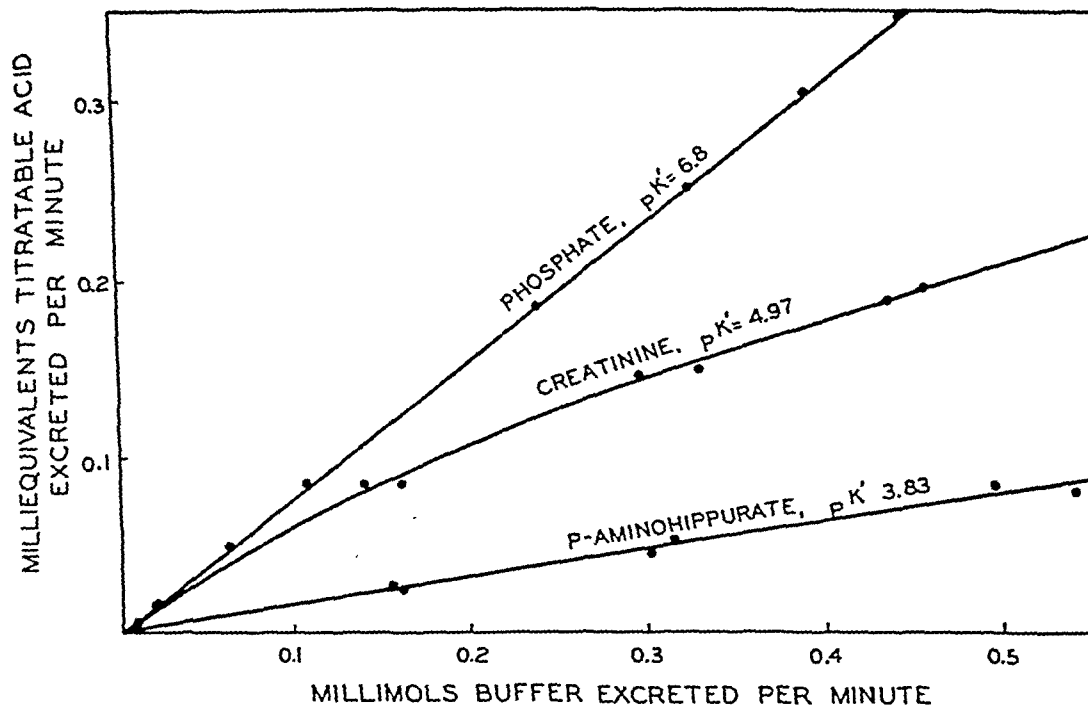


FIG. 2. A COMPARISON OF THE EFFECTIVENESS OF SEVERAL URINARY BUFFERS IN MAN IN ACIDOSIS

All data on subject R. F. P.

The relationship between the rate of excretion of titratable acid and the acid strength of the urinary buffer

Beta-hydroxybutyric acid is a relatively strong buffer acid, and in solutions of pH 4.7 half exists as free acid and half as neutral salt. In contrast, the second hydrogen of phosphate is only weakly acidic, and in solutions of this reaction, phosphate exists entirely in the titratable monobasic form. Since the reaction of the urine is limited to an acidity no greater than pH 4.5 to 4.7 it obviously follows that the stronger the buffer acid, the less completely can the renal tubules exchange hydrogen ions for ions of fixed base bound by that buffer in the tubular urine. Accordingly, when large quantities of beta-hydroxybutyrate are excreted in the urine in diabetic ketosis, sufficient base is lost to deplete the body reserves and lead to acidosis.

In Figure 2 is presented a comparison of the rates of excretion of titratable acid at a series of equivalent molar excretion rates of phosphate, creatinine and para-aminohippurate. The extent of the acidosis was essentially the same in the

3 experiments, for plasma bicarbonate concentrations varied only from 13 to 15 mM per liter. In each instance the rate of excretion of titratable acid was corrected by subtracting from the observed value that acid due to buffers other than the one specifically studied. For example, in the experiments on phosphate, the titratable acid due to the excretion of endogenous creatinine and organic acid was deducted from the total; in the experiment on para-aminohippurate, the titratable acid due to phosphate was also deducted. Such corrections are appreciable only at low rates of buffer excretion; they obviously account for the fact that all curves start at the origin. These corrections have been applied to the data presented in Figures 2, 3 and 4, but not to the data in Figure 1.

It is evident from Figure 2 that the rate of excretion of titratable acid at any given rate of excretion of buffer is determined by the acid strength of the buffer. Thus, secondary phosphate, the weakest acid buffer ($pK' = 6.8$), yields the greatest proportion of base in exchange for hydrogen ions; and para-aminohippurate, the strongest acid buffer ($pK' = 3.83$), yields the least proportion

of base in exchange for hydrogen ions. Creatinine, a buffer of intermediate strength ($pK' = 4.97$), occupies an intermediate position in the series. Beta-hydroxybutyrate ($pK' = 4.70$) would fit into this series between creatinine and paraaminohippurate somewhat closer to the former than to the latter. Primary phosphate is such a strongly acid buffer ($pK' = 2.11$) that the kidney can exchange hydrogen ions for the base which it binds only in insignificant trace amounts. Were they plotted in this diagram, such points would fall on or very near the base line.

The relation between the rate of excretion of titratable acid and the extent of the acidosis

It has been repeatedly observed that the ingestion of sufficient acid to depress the plasma bicarbonate level results in the prompt excretion of urine of low pH, and in the elimination of increased quantities of titratable acid (7). In Figure 3 are presented results of 2 experiments with creatinine, identical throughout except for the fact that in one a moderately severe acidosis had been induced, while in the other acid base balance was within limits of normal. It is evident at any given rate of excretion of buffer that the rate of excretion of titratable acid is greater in acidosis than when acid base balance is normal. What is surprising is the rather high rate of excretion of acid which is attained when the alkali reserve is within normal limits. Even more surprising are the rather small

differences in the rates of excretion of titratable acid in similar paired experiments in which phosphate was infused, cf. Figure 4. In the experiment in which no ammonium chloride was ingested, the plasma bicarbonate level was within the accepted range of normal (24.2 mM per liter), yet the infusion of phosphate was accompanied by increases in titratable acid excretion nearly the same as those observed in moderately severe acidosis. It seems reasonable to interpret these results as meaning that plasma bicarbonate concentrations within a range accepted as normal, characterize a state of mild acidosis so far as the kidneys are concerned. Furthermore, stimulation of the tubular exchange of hydrogen ions for sodium ions must be nearly maximum under such conditions of mild acidosis. Indeed, experiments to be presented in a subsequent paper on bicarbonate indicate that maximum stimulation of acid excretion is effected by reducing plasma bicarbonate to approximately 20 mM per liter.

A fact which is not evident from the data presented in these charts is that the excretion of increased quantities of buffer actually stimulates the formation of urine of more acid reaction. In the initial control periods of the experiment with phosphate in which the bicarbonate content of the plasma was 24.2 mM per liter, the pH of the urine was just below 7.0. Had this pH been maintained in subsequent periods in which the rate of excretion of phosphate was increased, the rate of excretion of titratable acid would have risen con-

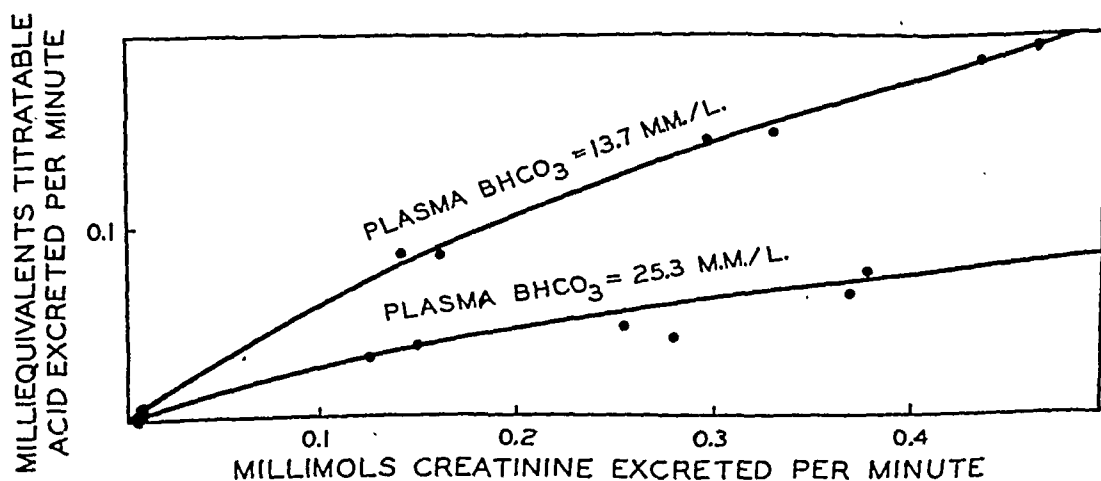


FIG. 3. THE RELATION BETWEEN THE RATE OF EXCRETION OF TITRATABLE ACID AND THE DEGREE OF ACIDOSIS AT A SERIES OF COMPARABLE EXCRETION RATES OF CREATININE

All data on subject R. F. P.

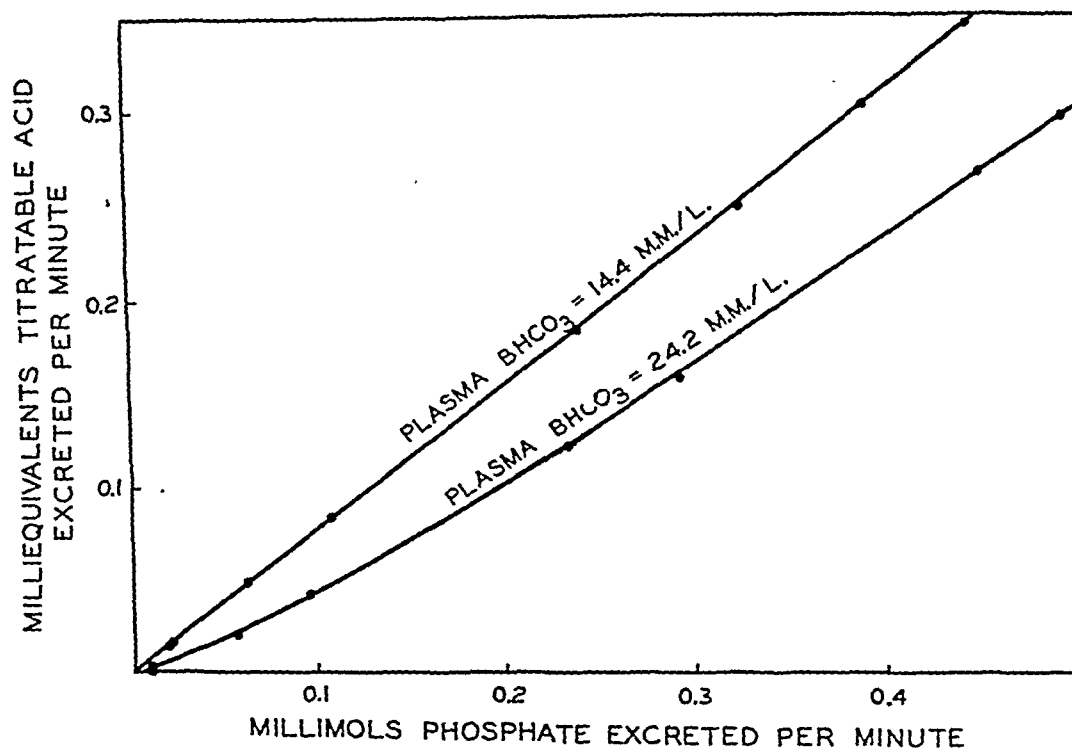


FIG. 4. THE RELATION BETWEEN THE RATE OF EXCRETION OF TITRATABLE ACID AND THE DEGREE OF ACIDOSIS AT A SERIES OF COMPARABLE EXCRETION RATES OF PHOSPHATE

All data on subject R. F. P.

siderably. But the urine actually became more acid during phosphate infusion until at the highest rate of phosphate excretion urine pH dropped below 6.0. The reason for this change in reaction of the urine on the infusion of phosphate is not known, but it has been observed previously in animal experiments (6, 8).

The effect of acidosis on the renal tubular reabsorption of phosphate

Phosphaturia is commonly observed in ammonium chloride acidosis and in the acidosis of diabetic ketosis (9, 10). The excess urinary phosphate is derived in large part from stores of non-diffusible organic acid soluble phosphorus within the cells, hydrolysis of which liberates inorganic phosphate into the plasma (11). According to 1 view, the renal reabsorptive mechanism for phosphate is unaltered in acidosis and the elevation of plasma phosphate which results from hydrolysis of the esters adequately explains the increased rate of phosphate excretion (12). On the other hand, it has been claimed that the plasma

phosphate concentration is below normal in acidosis and that the phosphaturia results from a depression in the capacity of the renal tubules to reabsorb phosphate (13). Since both views are based upon experimental work on dogs neither may be strictly applicable in man. We have accordingly analyzed our data in such a way that we might compare the characteristics of the reabsorptive mechanism in acidosis and in the normal, both at normal and elevated plasma concentrations of phosphate.

In Figure 5, rates of filtration, reabsorption and excretion of phosphate are plotted against the quantity of phosphate filtered per minute. All data were obtained in experiments on a single subject. The values designated as *acidosis* were observed following sufficient ammonium chloride to reduce the plasma bicarbonate concentration to 12 to 15 mM per liter. The values designated as *normal* were observed at plasma bicarbonate levels between 24 and 26 mM per liter. It is evident that as the quantity of phosphate filtered exceeded 0.20 mM per minute, the renal tubular reabsorptive

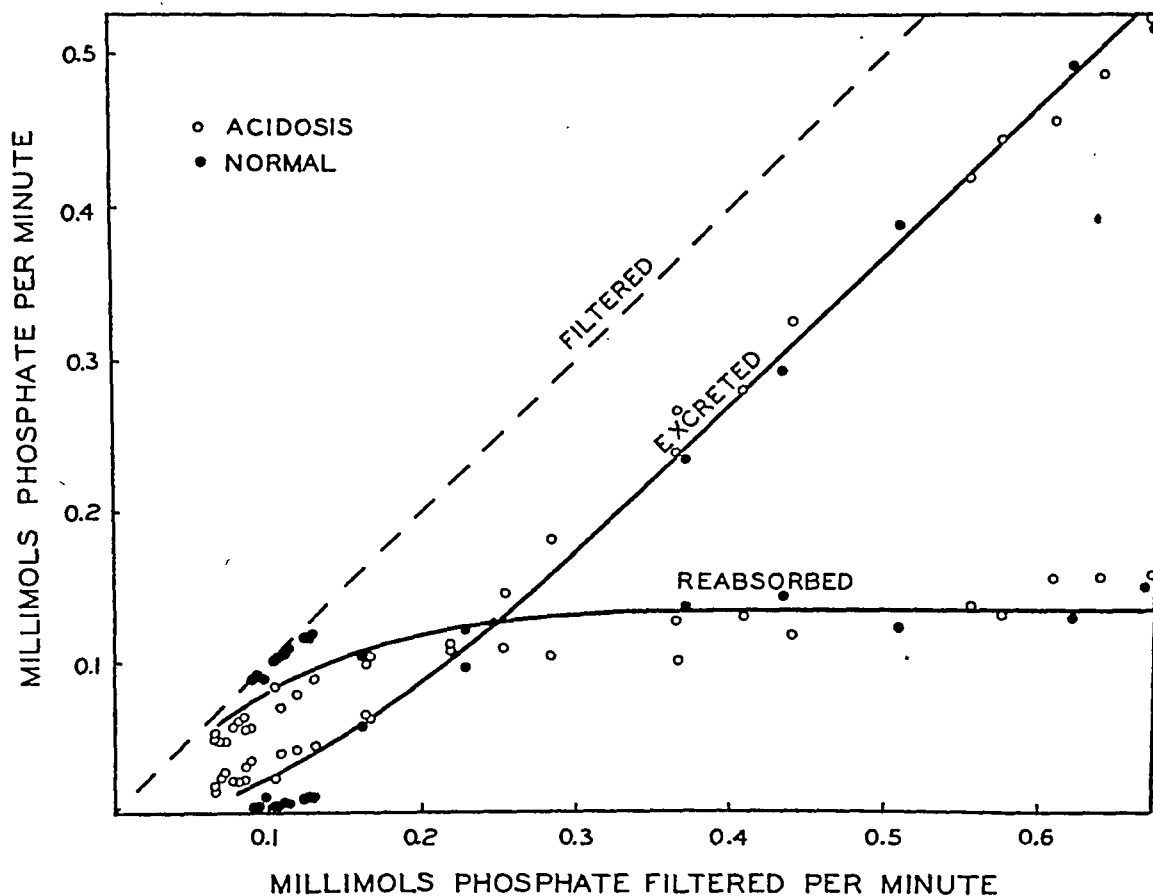


FIG. 5. THE RELATION BETWEEN THE QUANTITY OF PHOSPHATE FILTERED THROUGH THE GLOMERULI AND THE QUANTITIES REABSORBED BY THE RENAL TUBULES AND EXCRETED IN THE URINE

All data on subject R. F. P.

mechanism became saturated and phosphate was returned from tubular urine to blood at an average rate of 0.130 mM per minute. All phosphate filtered in excess of this quantity was excreted in the urine. There appears from our limited data to be no difference between this maximum rate of reabsorption in acidosis and under conditions of normal acid-base balance. In this respect, results on man and dog (12) are in agreement. In contrast, at normal plasma concentrations of phosphate the reabsorption of phosphate is significantly less complete in acidosis than under conditions of normal acid-base balance. Accordingly, the spontaneous rate of excretion of phosphate is higher in acidosis, a factor no doubt of major importance in explaining the phosphaturia in man. In our experience dogs show much less phosphaturia and no appreciable change in reabsorption of phosphate at normal plasma levels in acidosis (12). In these observations on man there appeared to be no significant effect of acidosis on plasma phosphate con-

centration, the range of variation being about the same in acidosis as in the normal. Apparently diminished reabsorption compensated more or less exactly for increased liberation of phosphate from intracellular stores. However it must be emphasized that the acidosis in our experiments was of short duration (24 hours). In more prolonged states of acidosis plasma phosphate may fall to levels below normal.

DISCUSSION

It is evident from the experiments presented above that a number of interrelated factors limit the quantity of titratable acid which the kidney can excrete. First, the kidney is limited in its capacity to excrete urine containing hydrogen ions in high concentration. This capacity undoubtedly varies somewhat in different normal subjects. Although the accepted limit of acidity is pH 4.7 (3), 2 of our 4 subjects could excrete urine as acid as pH 4.49. Expressed as a hydrogen ion

gradient between urine and plasma, the maximum which the renal tubules of these 2 subjects could establish is 800 to 1. It is obvious that in the absence of buffer the exchange of hydrogen ions for ions of fixed base in the tubular urine could proceed only to an insignificant extent before this limiting gradient would be attained. Second, the quantity of buffer which is eliminated by the normal subject is rather small, and as a consequence the quantity of base which can be salvaged from the urine by hydrogen ion exchange is likewise small. In the normal individual sufficient buffer (largely phosphate) is present in the urine to permit recovery of only 10 to 30 mEq of base each day. In contrast, in diabetic ketosis as much as 500 to 1000 mM of beta-hydroxybutyrate and aceto-acetate may be excreted in 24 hours. Because of the increased availability of buffer, up to 150 mEq of base may be returned to the body each day in exchange for hydrogen ions. Third, the acid strength of the buffer is a limiting factor in the exchange of hydrogen ions for base. Obviously the lower the acid strength, the more completely can the kidney salvage base bound by buffer in urine of maximal acidity. All of the secondary base can be recovered from phosphate, only half can be recovered from beta-hydroxybutyrate. Although titratable acid excretion in diabetic ketosis may increase to some 10 times the normal because of increased buffer excretion, the loss of available base is likewise increased because of the high acid strength of the buffer. Theoretically at least there must be some ceiling to the quantity of hydrogen ions which the kidney can exchange, even under hypothetical conditions of unlimited supplies of buffer of optimum acid strength, for the process requires energy, and the energy available to the kidney is not unlimited. However, in our experiments we have by no means approached any such limiting rate of exchange, although for short periods of time during phosphate infusion hydrogen ions have been transferred at a rate which would be equivalent to 0.6 mol per day. This is equal to the excretion of 6000 ml. of one-tenth normal acid, or to the renal manufacture from a buffer precursor of 50 grams of sodium bicarbonate per day.

As Henderson (14) has so clearly pointed out, the properties of secondary phosphate are such as to render it the ideal urinary buffer. However,

from this fact one must not make the extrapolation that the phosphaturia of diabetic acidosis is compensatory in nature. The kidney can salvage only the secondary base of phosphate, the primary bound base is lost in the urine. Thus, for each extra mol of phosphate excreted, the body store of base is depleted to the extent of 1 equivalent. The only point about the process which can be considered as compensatory is the fact that the hydrolysis of organic intracellular phosphate enables base of the cells to be transferred to the plasma. Thus, the cellular phosphate esters bind roughly the same quantity of base per mol of phosphate as is bound by inorganic phosphate in the plasma. The excretion of this phosphate in inorganic form as monobasic phosphate returns to the plasma the secondary bound base as bicarbonate. Thus, intracellular base replenishes extracellular bicarbonate in acidosis, but in the process more than half the base is lost. The kidney actually operates a little more efficiently on beta-hydroxybutyrate than it does on phosphate, for in severe ketosis less than half the base bound by this hydroxy acid in the plasma is lost in the urine.

The reabsorptive mechanism for phosphate in man appears to be qualitatively similar to that in the dog in that the tubules are capable of reabsorbing only a limited quantity of phosphate per unit of time. When as a consequence of an increase in plasma concentration the quantity delivered into the filtrate exceeds the reabsorptive capacity of the tubules, the excess is excreted in the urine. The reabsorptive capacity is fixed, limited, and independent of plasma concentration when that concentration is sufficiently high to saturate the mechanism. This maximum reabsorptive capacity appears to be unaffected in either dog or man by moderately severe acidosis of short duration. However, at low plasma concentrations the kinetics of the reabsorptive process appear to be altered in man, for reabsorption is only partial and excretion is correspondingly increased. The diminished reabsorption at normal plasma concentrations is undoubtedly a factor in explaining the phosphaturia of acidosis in man. Apparently, in our experiments increased excretion was balanced more or less exactly by increased delivery of inorganic phosphate into the plasma, for we have observed no significant differences in plasma level in man as a consequence of acidosis.

CONCLUSIONS

The rate of excretion of titratable acid in man as in the dog is largely determined by 3 factors, namely: (1) the rate of excretion of buffer; (2) the acid strength of the buffer; and (3) the extent of the reduction in the plasma concentration of bicarbonate. For phosphate and para-aminohippurate the relationship between buffer and titratable acid excretion is essentially a linear one within the range of buffer excretion that we have studied. For creatinine the relationship is curvilinear because urine pH increases slightly at high rates of buffer excretion. At any given molar rate of excretion of buffer the rate of elimination of titratable acid is highest for the buffer of lowest acid strength. The accepted normal range of 24 to 26 mM of bicarbonate per liter of plasma constitutes a state of mild acidosis so far as the kidneys are concerned, and titratable acid elimination proceeds at a nearly maximal rate when a buffer of favorable acid strength is presented to the kidney. Further reduction of plasma bicarbonate to 20 mM per liter maximally stimulates the acid excretory mechanism. The results presented are in accord with the view that the urine is acidified by the exchange of hydrogen ions formed within the tubular cells for ions of fixed base in the tubular urine.

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EXCHANGES OF SODIUM AND POTASSIUM IN FAMILIAL PERIODIC PARALYSIS¹

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The constant association of a fall in concentration of serum potassium with attacks of muscular weakness, plus the relief of the weakness by the administration of potassium salts, have clearly indicated that familial periodic paralysis involves a defect in potassium metabolism. The nature of this is not completely understood.

Following the observation that the concentration of potassium in serum was lowered at the time of paralysis (1, 2), a number of other investigators studied potassium exchanges in this condition to ascertain the route of removal of potassium from serum and extracellular fluid. It has been found that the urinary excretion of potassium prior to and during attacks of paralysis, with but 1 exception, was minimal, and hence the lowered concentration of this cation must result from a shift into cells (3 to 7). Only during a water diuresis preceding an attack did Gammon and co-workers (6) find an increased excretion of potassium in the urine. It has been suggested that an abnormal demand for potassium in muscle cells is present in this condition (7). The production of lowered concentrations of serum potassium and of paralysis by the administration of carbohydrate, insulin, or epinephrine has also led to the hypothesis that potassium may be moved from extracellular fluid into cells of the liver or other tissues of the body during glycogenesis or carbohydrate combustion.

Ferrebee, Gerity, Atchley, and Loeb (8) have reported the results of an extensive metabolic study of a patient with this disease. They confirmed the finding that potassium excretion diminished coincidently with the fall in serum concentration during an attack, and found erratic fluctuations in excretion of potassium and sodium between attacks. They did not, however, quantitate the exchanges of potassium and sodium between

the extracellular and intracellular phases of the body fluids. This has been done, therefore, in the studies reported in this paper in the hope that the magnitude and the direction of the exchanges of these cations might help further to elucidate the abnormality of potassium metabolism which is present in this disease.

EXPERIMENTAL PROCEDURE AND METHODS

The patients studied were 2 of the 3 cases of familial periodic paralysis which have been diagnosed in the New Haven Hospital since 1921. Patient R. B. was a 29-year-old white man who had had attacks of muscular weakness every 2 to 4 weeks since the age of 18 years. One sister was subject to similar attacks. Patient D. D. was a 34-year-old white man who had had intermittent attacks of paralysis since the age of 15 years. One half-brother was said to have similar but less severe symptoms. In both patients the attacks usually began during sleep in the early morning hours and were relieved by ingestion of potassium. On admission and during the attacks the patients' physical examinations were negative except for motor weakness and diminished to absent deep reflexes.

The quantitative data on Patient R. B. were obtained during recovery from a spontaneous attack of generalized paralysis. In the first period of 3 hours KCl was given by mouth; in the succeeding period of 16 hours the patient took food and water. In Patient D. D. the quantitative study extended over a period of 4 days, during which an attack of paralysis was induced by withdrawal of the maintenance dose of KCl and the administration of large doses of carbohydrate. The patient received no other food during the entire study. Recovery was effected by giving KCl intravenously and orally.

The patients were weighed and blood was taken for analysis at the beginning and end of each period. Concentrations of Cl, Na, and K in serum were determined in both subjects as were Cl, Na, and K balances. In Patient D. D., there were measured as well nitrogen balances and also the concentrations in blood of nonprotein nitrogen and of sugar.

The electrolyte content of food eaten by the first subject was calculated by means of Sherman's tables (9); the milk and lemon juice present in the sucrose-lactose drink taken by Patient D. D., and a stool which he passed following oral KCl, were analyzed for Na, K, Cl, and nitrogen.

In Patient R. B. sodium in serum, and potassium in

¹ Aided by a grant from the Fluid Research Fund of Yale University.

² Deceased, June 26, 1947.

serum and urine were determined by the method of Hald (10); sodium in urine, by the method of Butler and Tut-hill (11). In the studies on Patient D. D., sodium and potassium concentrations were measured by means of the flame photometer (12). The whole blood, milk, and stool were first ashed at 550° to 650° C. The methods used for the determination of chloride, blood sugar, non-protein nitrogen, urine nitrogen, the relative blood cell volume, the volume of distribution of mannitol, and the water content of serum and whole blood have been listed in previous publications (13, 14).

METHOD OF CALCULATION

The method of calculation has been detailed in previous publications (13, 15, 16). The total water balance was calculated from the change in weight corrected for the solids lost and from the metabolic mixture. The change in extracellular fluid volume was calculated from the balance of chloride and the changes in its concentration in serum. In Patient D. D. the volume of the extracellular fluid was measured by the volume of distribution of mannitol³ in the middle of the experiment. The change in chloride space was then calculated from this value both forward and backward in time instead of assuming an initial extracellular volume. Changes in intracellular fluid volume were taken as the difference between the volumes of total water and extracellular fluid.

The changes in, or balance of intracellular sodium and potassium, which were independent of the building up or breaking down of tissues (in excess of nitrogen), were taken to be the balances of these 2 ions remaining after the total balances were corrected for increments or decrements associated with the extracellular fluid and with the balance of protein. The change in total osmotically active base is taken to be the discrepancy between the theoretical balance of sodium and potassium, calculated from the water balance and the change in concentration of serum sodium, and the observed total balance of sodium plus potassium.

RESULTS

The analytical and derived data are given in Tables I, II, and III, and in Figure 1.

Exchanges of potassium. In Patient R. B., during the immediate period of recovery produced by the oral administration of KCl, potassium was taken up into both extracellular and intracellular phases, and the extracellular concentrations of potassium rose to a normal level. During the subsequent 16 hours during which little potassium was given, the extracellular potassium was retained, but the cellular increment for the most part was lost in the urine.

In Patient D. D., during the first 26 hours of

the experiment following the withdrawal of KCl and the ingestion of large doses of carbohydrate, the loss of potassium in the urine resulted in a small negative potassium balance. Most of this potassium was cellular. During the subsequent period from hours 26 to 62, at which time the peak of paralysis was reached, this negative balance persisted, but the deficit of cell potassium which had developed during the period of hours 0 to 26 was cancelled by transfer of potassium from the extracellular space. This transfer of potassium from the extracellular to the intracellular phase was associated with the associated decline in extracellular potassium concentration to 2.2 m.eq. per liter coincident with the onset of muscular paralysis.

Following the intravenous administration of KCl (hour 62 to 68) a moderately large amount of potassium entered the intracellular phase but the extracellular potassium deficit was only partially cancelled and some paralysis persisted. When more KCl had been given orally (hours 68 to 74), the balance of extracellular potassium became positive, both the concentration and the amount of extracellular potassium increased, and simultaneously the muscular weakness disappeared. At the end of the study (hour 98) the amount of extracellular potassium was approximately the same as it had been initially, but the amount of intracellular potassium had increased to a moderate degree, relative to the start of the experiment.

Exchanges of sodium. In Patient R. B., during recovery some sodium shifted from the extracellular to the intracellular phase at the same time that potassium was being taken up into both phases. In Patient D. D. there appeared to be a rough reciprocal relationship between sodium and potassium, the amount of cellular sodium increasing slightly during the initial diminution of cellular potassium, and an opposite relationship obtaining during the recovery period when potassium entered cells.

Potassium and sodium in erythrocytes. In Patient D. D. the potassium and sodium contents of red blood cells were determined at the peak of paralysis (hour 62) and again following recovery (hour 74). A slight increase in the concentrations of sodium and potassium in water of red cells occurred during this interval. At both times, however, values were still below normal (17).

³ The mannitol was supplied through the courtesy of the Medical Research Division of Sharp and Dohme, Inc.

SODIUM AND POTASSIUM EXCHANGES IN PERIODIC PARALYSIS

TABLE I
Periodic paralysis
External exchanges of water, electrolytes, and nitrogen

Periodic paralysis																								
External exchanges of water, electrolytes, and nitrogen																								
Patient	Time from start of experiment	Paral-ysis	Body weight	Intake*						Output												Balance		
				H ₂ O	Carbo-hydrate	Cl	Na	K	N	Urine						Stool	Cl†	Na†	K	N				
										Vol.	Cl	Na	K	N	grams						m. eq.	m. eq.	grams	
																								ml.
R. B.	0	++	62.5																					
	2	0	62.5	500	134	218§	134	398§		75	14.4	11.0	1.9		100	+117	-14	+132						
	18	0	61.6	Ad lib.	20§§	248§	218§	398§		1270	170.0	88.0	136.0		50	-148	-69	-97						
D. D.	0	0	76.9	3000	240		0.8	1.2	0.06	2240	39.9	27.6	33.2	4.53		-43	-31	-32						
	5	0	77.1	3100	240		0.8	1.2	0.06	2965	27.3	20.8	31.4	6.28	20	-30	-23	-30				-4.47		
	26	0	76.1	5900†	500	11.8	9.6	15.3	2.11	5490	26.9	23.6	23.6	6.75	160	-18	-18	-8				-6.22		
	50	+	75.9	2800	336		1.1	1.7	0.09	1700	10.6	3.1	2.9	2.65		-14	-6	-1				-4.64		
	57	+	76.8	500	60		0.2	0.3	0.02	565	8.5	1.3	1.4	1.25		-11	-5	-1				-2.56		
	62	+	76.7	2150§	108§	134§		134§		1420	12.2	1.7	2.8	2.02		+118	-7	+131				-1.23		
	68	+	77.4	600**	134**	134**		134**		435	28.9	5.3	25.9	1.57		+91	-22	+108				-2.02		
	74	0	76.6	2700††	49††	163.5††	20.0††	166.2††	4.97††	2555	247.0	47.7	279.0	6.82	130††	+91	-37	-113				-1.72		
	98	0	74.8																			-1.85		

* Intake is by mouth except where otherwise indicated.
† Balances include a small amount of Cl and Na lost in serum drawn for analysis.
§ 10 grams KCl given intravenously in 5 per cent glucose.
** 10 grams KCl given intravenously in 5 per cent glucose.
†† 10 grams KCl given intravenously in 5 per cent glucose.

* Intake is by mouth except where otherwise indicated.
† Balances include a small amount of Cl and Na lost in serum drawn for analysis.
‡ Includes 400 ml. of whole milk.
§ 10 grams KCl given intravenously in 5 per cent glucose solution.
¶ 10 grams KCl given by mouth.
‡‡ Includes 1000 ml. whole milk and 10 grams of KCl.
§§ Liquid stool containing 10.8 m. eq. Cl, 11.8 m. eq. Na, 8.9 m. eq. K, and 0.15 gram N; the other stools in both experiments were formed and were not analyzed.

§§ Includes 600 ml. of milk and 4 eggs.
Balance data are expressed per individual period rather than cumulatively. Time from start of experiment indicates end of period at which time serum analyses were made and balances for period determined.

Time from start of experiment indicates end of period at which time serum analyses were made and balances for period determined.

TABLE II
Periodic paralysis
Analyses of blood, serum, and red cells

Patient	Time from start of experiment	Paralysis	Blood				Serum				Red cell H ₂ O	
			NPN	Sugar	Relative cell vol.	Hemo-globin	H ₂ O	Cl	Na	K	Na	K
	hours		mgm. per cent	mgm. per cent	per cent	grams per cent	grams per liter	m. eq. per liter	m. eq. per liter	m. eq. per liter	m. eq. per liter	m. eq. per liter
R. B.	0	++					*	96.0	141.6	2.3		
	2	0					*	100.8	127.7	4.4		
	18	0					*	94.6	129.1	4.4		
D. D.	0	0	38	76			938	103.8	144.8	4.3		
	5	0	31	71			936	104.0	143.5	4.4		
	26	0	28	84			932	102.7	146.9	4.0		
	50	+	31	80			929	106.4	148.0	3.3		
	57	+	27	80			927	102.6	144.8	2.9		
	62	++	28	78	45.8	16.6	936	103.0	147.0	2.2	.0	116
	68	+	28	73			932	109.0	146.5	3.5		
	74	0	28	68	40.9	15.6	942	107.8	144.3	6.1	6.5	122
	98	0	32	—			939	106.6	142.0	4.8		

* Water of serum[†] was assumed to be 930 grams per liter.

Exchanges of water. Changes in volume of total body water and of extracellular and intracellular fluid were essentially negligible. In Patient D. D. the volume of distribution of mannitol was determined after recovery (hour 74). At this point the mannitol space, which appears to meas-

ure the volume of extracellular fluid (14), was found to be 16.4 liters, or 21.4 per cent of the body weight. Calculating backward by means of the chloride balance, the extracellular fluid volumes at the peak of paralysis (hour 62) and at the beginning of the experiment (hour 0) were equiv-

TABLE III
Periodic paralysis
‡ Calculated exchanges of water, sodium, and potassium between the extracellular and intracellular phases of body fluid

Patient	Time from start of experiment	Paralysis	Change in volume of			Na and K balances for				Change in total osmotically active base
			Total body water	Extra-cellular fluid	Intra-cellular fluid	Extracellular phase		Intracellular phase in excess of N		
						Na	K	Na	K	
	hours		liters	liters	liters	m. eq.	m. eq.	m. eq.	m. eq.	m. eq.
R. B.	0	++								
	2	0	+0.1	+0.4†	-0.3	-123	+29	+109*	+103*	-758
	18	0	—	-0.5†	—	-47	-2	-22*	-95*	+116
D. D.	0	0								
	5	0	+0.3	-0.5	+0.8	-88	-1	+57	-29	+43
	26	0	-0.7	-0.1	-0.6	+49	-6	-71	-13	+163
	50	+	±0	-0.5	+0.5	-79	-13	+62	+20	+76
	57	+	+0.9	+0.4	+0.5	+15	-5	-21	+5	-3
	62	++	±0	±0	±0	+13	-12	-18	+15	+46
	68	+	+0.8	±0	+0.8	+2	+22	-9	+114	+6
	74	0	-0.5	+1.1‡	-1.6	+103	+46	-125	+66	-326
	98	0	-1.3	-0.6	-0.7	-120	-24	+84	-79	-150

* These values represent the balance for the total intracellular phase (b_{Na_i} and b_{K_i}) uncorrected for loss of nitrogen.

† Calculated from an initial extracellular volume (E_1) taken to equal 20 per cent of the body weight.

‡ The volume of distribution of mannitol at this time was determined to be 16.4, 15.6, and 16.7 at 1, 1½, and 2 hours, respectively, following the intravenous injection of 25.45 grams of mannitol.

Balance data are expressed per individual period rather than cumulatively. Time from start of experiment indicates end of period at which time balances for the period were determined.

SODIUM AND POTASSIUM EXCHANGES IN PERIODIC PARALYSIS

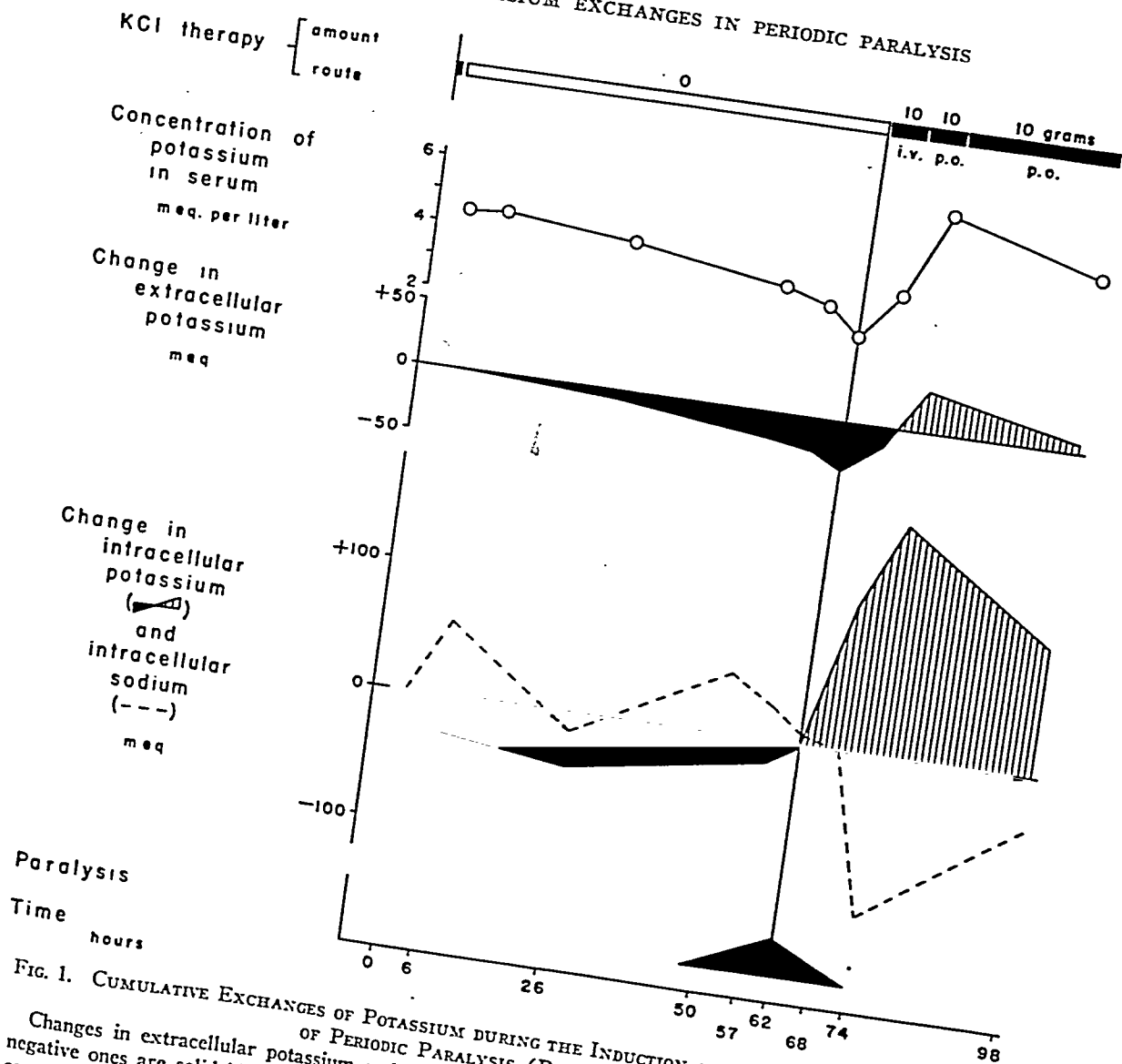


FIG. 1. CUMULATIVE EXCHANGES OF POTASSIUM DURING THE INDUCTION AND TREATMENT OF AN ATTACK OF PERIODIC PARALYSIS (PATIENT D. D.)

Changes in extracellular potassium and intracellular potassium in excess of nitrogen are shown; the negative ones are solid black, the positive ones are lined vertically. The concentration of potassium in serum is plotted with open circles. The change in intracellular sodium is plotted with a broken line. The presence of paralysis is indicated by a solid black triangle at the bottom of the figure. The data indicate that during the actual onset of paralysis, potassium moved from the extracellular to the intracellular phase. During treatment with KCl, a positive balance of intracellular potassium was built up before the extracellular deficit was overcome. The change in cell sodium showed an inverse relationship to that of cell potassium.

alent to 20.0 and 21.1 per cent of the body weight, respectively. These values are of the order of magnitude of those accepted as normal for the extracellular phase (18). The change in plasma volume which occurred between the peak of paralysis (hour 62) and recovery (hour 74) amounted to a relative increase of 17 per cent, as estimated from the hematocrit

values and hemoglobin concentrations (19). This rise in plasma volume is not proportional to the increase of 1.1 liters in extracellular fluid (7 per cent), and hence suggests some redistribution of water between plasma and interstitial fluid during paralysis.

Changes in total osmotically active base. The discrepancies between the actual total balances of

sodium plus potassium and the balances of total osmotically active "base" in the body, as calculated from the changes in volume of body water and extracellular sodium concentration, probably represent changes in osmotic activity of base⁴ within body cells (16). During the early period of depletion of cell potassium in Patient D. D., cell base appeared to have been activated. Conversely, during the recovery period when cells were taking up potassium, a large decrease in the osmotically active components of cells occurred in both patients.

Electrocardiographic alterations. In Patient R. B. the T waves which were absent during paralysis returned and the minor changes in S-T segments disappeared during recovery (hours 2 and 18). In Patient D. D. the inverted T wave in lead 2 which was present during the paralysis reverted to an upright position at the time of partial recovery and to normal height in the upright position at the time of complete recovery. These changes are consistent with the known effect of low concentrations of serum potassium on the electrocardiogram (20).

DISCUSSION

Interpretation of the potassium transfers. These data clearly indicate that the movement of potassium immediately associated with the onset of muscular paralysis in this disease consists of a transfer of extracellular potassium into the intracellular phase. The result of this transfer is a striking depression of the extracellular concentration. It is not possible to identify, however, the portion of the cellular phase into which this potassium went. The balances as calculated are only for the overall phases of the intact organism and do not differentiate the distribution of electrolytes in the various tissues. Speculation on this point based on indirect evidence, however, is possible. The first possibility is that potassium has entered the cells of skeletal muscle which constitute the largest depot of potassium and the major portion of the intracellular phase. This site of the transfer is suggested by the experimental conditions which

have produced diminution of extracellular potassium and paralysis in this disease. These conditions have been the administration of carbohydrate, of insulin, and of epinephrine (7). All of these substances are known to produce a lowering of serum potassium concentration (21 to 26), and all of them are associated with increased oxidation of carbohydrate in the muscles. It would seem reasonable, therefore, to ascribe the transfers of potassium which take place in periodic paralysis to the cycle of carbohydrate combustion in skeletal muscle. An alternate possibility, that the extracellular potassium shifts into the cells of liver in association with glycogenesis (27), is unlikely in view of the fact that insulin and epinephrine are predominantly glycogenolytic substances. Both D'Silva (28) and Gerschman (26) have shown that the transient initial rise in the concentration of serum potassium following the administration of epinephrine is abolished when the liver is excluded or removed. Epinephrine apparently promotes the transfer of potassium with glycogen from the liver to the muscles.

Whether or not the movement of extracellular potassium is into cells of skeletal muscle or into those of other tissues, there must be some difference between these patients and the normal individual with respect to replacement of the extracellular potassium deficit. One explanation would be that all the body cells of the patient with periodic paralysis contain a normal or an excessive amount of potassium owing to an abnormal avidity for this cation. Such an avidity, however, can hardly be explained by the binding of potassium to protein or organic phosphate complexes within the cell. The evidence in these experiments indicates that during the development of paralysis the osmotic activity of cell fluid is increased and conversely during recovery, is decreased. Besides, analysis of the relation of exchanges of sodium and potassium across the cell membrane to changes in osmotic activity of base within the cell, shows that there is little quantitative relationship between the two (29).

Another possibility is that the replacement of the extracellular potassium is prevented by a chronic deficit of muscle potassium. This is suggested by the fact that following treatment of Patient D. D.'s paralysis with intravenous KCl (hour

⁴ This refers to the end-result, and does not exclude the possibility that the process is initiated by alterations in the osmotic activity or the base-binding capacity of cellular anions.

68), 114 m.eq. of potassium had been taken up into the intracellular phase while a deficit of 15 m.eq. of extracellular potassium persisted and muscular weakness was still present. Unequivocal evidence for such a state of muscle potassium depletion is missing but suggestive evidence is as follows: Darrow (30) has analyzed muscle obtained at autopsy from 1 case and found the potassium content to be low. The muscle, however, was atrophic and the changes found were probably related to the postmortem state or the atrophy rather than to the periodic paralysis. Erythrocyte content of potassium has been shown to be lower than normal by other workers (4, 7) as well as in this study. Finally, the positive balance of 100 m.eq. of cell potassium in Patient D. D., at the end of the study, might suggest that the patient had a cell potassium deficit at the beginning of the experiment. However, these are all conjectural interpretations and it is very difficult to establish their validity, particularly since this balance represents an increase of only 1.3 m.eq. per kilogram of body weight, a relatively minor increment in muscle cell potassium.

Pathogenesis of the paralysis. Certain conclusions can be drawn from the quantitative data obtained concerning the relation of the potassium exchanges to the muscular paralysis. The paralysis cannot be directly due to an increase in potassium content of the muscle cells in general because of the relatively minute amount of potassium transferred from the extracellular fluid with the onset of paralysis. Depletion of muscle cell potassium could contribute to the mechanism only indirectly by preventing replacement of the extracellular potassium deficit. The paralysis must be due either to the depletion of extracellular potassium with the lowered concentration or to the presence of the displaced potassium in a special tissue. It is hard to see, however, how the latter disturbance would be relieved by administration of more potassium. The primary cause of the paralysis, therefore, probably lies in the loss of potassium from the extracellular fluid, with the low concentration producing an effect on the neuromuscular mechanism. The difficulty with such a simple explanation is the fact that paralysis is not always present with hypopotassemia in other conditions (31 to 33), nor does it correlate exactly with

the fall in extracellular potassium concentration in this condition (7).

Specificity of the potassium defect. Depletion of extracellular potassium in association with the development of paralysis is certainly the chief physiological defect in this disease. However, this mechanism of paralysis is apparently not unique to this disease as the concurrence of hypopotassemia and muscular weakness has been reported in other clinical and experimental conditions. Ferrebee and co-workers (34) have shown that the administration to dogs of desoxycorticosterone, when potassium was withheld, produced deficits of muscle potassium ranging from 35 to 66 per cent of the initial content; replacement of the cell potassium by sodium; lowering of the extracellular potassium concentrations; and muscular weakness and paralysis. Holler (35) described muscular paralysis associated with hypopotassemia in a case of diabetic acidosis undergoing treatment with large amounts of glucose and insulin. It is well known that deficits of cellular potassium occur in this condition (36, 37). A similar association of muscular weakness with hypopotassemia has been reported in chronic nephritis (38) and in sprue (39).

In these other conditions the existence of deficits of cellular potassium has been established. Whether or not similar deficits exist in familial periodic paralysis, and what role they play in the disease is not known. The common denominator, however, appears to be the diminution in concentration of extracellular potassium.

SUMMARY

1. Potassium, sodium, and water exchanges were studied in 2 patients with periodic paralysis.
2. During the development of paralysis, potassium shifted from the extracellular to the intracellular phase with a resultant sharp decline in the extracellular potassium concentration.
3. During recovery large amounts of administered potassium were taken up by the intracellular phase before the extracellular potassium was replenished.
4. Reciprocal transfers of cell sodium were observed in 1 case but not in the other.
5. No significant changes occurred in the volumes of total body water and extracellular fluid.

6. Cellular processes which might effect the transfer of potassium are discussed.

7. It is concluded that the diminution in extracellular concentration of potassium is responsible for the development of paralysis.

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TRANSFERS OF CELL SODIUM AND POTASSIUM IN EXPERIMENTAL AND CLINICAL CONDITIONS¹

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In recent years much interest has been manifested in transfers of cellular electrolytes in various disease states. Loss of the main cation, potassium, in "excess" of nitrogen has been found in fasting (1), diarrhea (2), diabetic coma (3, 4), and dehydration (5). It has further been shown that at least partial replacement of deficits of cell potassium by increments of extracellular sodium occurs in animals on low potassium-high sodium diets (6, 7), in animals treated with desoxycorticosterone (8), in subjects with alkalosis (9, 10) and in some infants with diarrhea (11). Furthermore, the administration of potassium as well as sodium and water has been found to be therapeutically advantageous in some of these conditions (4, 12).

Not only do extensive transfers of electrolytes across the cell boundaries take place, but alterations in osmotic activity of the constituents of the cells occur as well (13 to 15). Presumably this is due to changes in the degree of dissociation of the cations bound with protein, phosphates, or other organic complexes. The conditions governing such phenomena and their relation to transfers of cations across the cell membrane are not known.

In this paper data are analyzed from a large number of experiments in dogs and human subjects with various alterations of body water and electrolytes. It is our purpose to identify factors which condition transfers of cations, to seek relationships between sodium and potassium during movements into and out of cells, and to calculate the osmotic adjustments which accompany such exchanges.

EXPERIMENTAL MATERIAL

Animal studies. The experimental data used in these analyses have been reported previously in a series of papers (16 to 20). Body fluids were altered as follows:

¹ Aided by grants from the Fluid Research Fund of Yale University.

² Deceased, June 26, 1947.

(1) hypertonicity and an increased concentration of sodium in body fluids were produced by either (a) injection of 2 to 6 per cent sodium chloride solution or (b) dehydration of the subject by means of urea diuresis or prolonged water deprivation; (2) hypotonicity and a lowered concentration of sodium were produced by either (a) intraperitoneal injection and subsequent withdrawal of glucose solution or (b) infusion of glucose solution into nephrectomized animals.

Reverse movements of body water were subsequently induced in 3 of these 4 groups. This was accomplished by giving water to dehydrated subjects (group 1b above), or sodium chloride to those with a deficit of sodium or an excess of water (groups 2a and 2b above). By these procedures the volume of intracellular water, which was decreased in the hypertonic animals and increased in those with hypotonicity, was restored partially or completely to normal.

Human studies. Data presented elsewhere (21) on subjects with or without food while on a partially or totally restricted intake of fluid are analyzed in this paper for evidences of transfers and inactivation of body base. In addition, data are used from experiments involving alterations in body water and electrolytes derived from 2 patients with attacks of periodic paralysis treated with potassium chloride (22); from another with congestive failure and anasarca; and a fourth with cirrhosis and ascites. These are used together with the experimental findings in 2 normal subjects (23). One of these took 240 grams of carbohydrate together with 20 grams of potassium chloride, and the other took large volumes of physiological saline during intervals of 4 and 6 hours, respectively.

The experimental procedures and the chemical methods have already been described (5, 15, 16).

Calculation of changes in cellular sodium and potassium. On the assumption that the chloride in extracellular fluid is not increased or decreased by movements of this ion out of or into cells, the change in cellular sodium (Na_i') or potassium (K_i') which has taken place in "excess" of protein or nitrogen may be calculated as follows:

$$\begin{aligned}\Delta\text{Na}_i' &= b_{\text{Na}} - \Delta\text{Na}_E - \Delta\text{Na}_P \\ \Delta\text{K}_i' &= b_{\text{K}} - \Delta\text{K}_E - \Delta\text{K}_P\end{aligned}$$

where

b_{Na} and b_{K} = balances of Na and K.
 ΔNa_E and ΔK_E = changes in Na and K in the extracellular fluid (E), as measured by the chloride space.

ΔNa_p and ΔK_p = changes in cellular Na and K associated with the catabolism or anabolism of protein. These are calculated on the basis that each kilogram of water-free muscle protein in the normal dog contains 7 m.eq. of sodium and 380 m.eq. of potassium (5, 16). These values are only slightly different in human muscle.

Calculation of changes in osmotically active cell base. The osmotically active cell base is taken to be that portion of sodium and potassium in cells which is not bound to other cellular constituents, such as the proteins and the phosphate complexes. This portion is, therefore, ionized and exerts an osmotic force. Variations in its magnitude induce movements into and out of cells in response to osmotic forces.³

The change in the amount of osmotically active base is taken to be the difference between the predicted balance of base (b_B) and the observed balances of sodium and potassium (b_{Na+K}). The former is calculated:

$$b_B = W_2 B_2 - W_1 B_1$$

where

B_1 and B_2 = initial and final concentration of base in the body fluids.

W_1 and W_2 = initial and final volume of total body water.

The predicted base balance rests on the change in body water and the assumption of complete osmotic equilibrium, and has no relation to the chloride space.

RESULTS

1. Transfers of potassium

It is apparent from Figure 1 that in dogs and humans under a variety of experimental conditions which alter greatly the water and electrolytes in the various body fluid compartments, potassium left cells far more often than it entered.

Cellular potassium decreased in a wide variety of experimental conditions, none of which appeared to be an indispensable prerequisite to such a change. These decrements were observed most often, however, in hypertonicity and dehydration produced by water restriction or urea diuresis (small solid circles, Figure 1). The potassium in cells declined significantly—i.e., more than 10 m.eq. in dogs and more than 25 m.eq. in humans—in 26 out of 44 experiments of this type. Hypertonicity induced by the injection of sodium chloride solutions, on the other hand, was associated

with a loss of cell potassium in only 6 out of 19 studies (large closed circles). Hypotonicity resulting either from salt depletion or injection of water into animals without renal function (open circles, small and large, respectively) was accompanied by a decrease in cellular potassium in only 9 out of 37 periods.

It is to be noted, furthermore, that these decreases in the cell potassium occurred without comparable increases of potassium in the extracellular fluid. The dog with anoxemia in this series (half-closed circle, Figure 1) with a large rise in serum potassium exactly equivalent to the drop in cell potassium is an exception to the general observation. This animal, however, was in an agonal state, and ordinary physiological forces and processes were no longer operative. In general, therefore, potassium which left cells merely passed through the extracellular fluid to be excreted.

However, when some profound interference with the renal mechanism, circulatory or anatomical, was present, potassium did not leave the cells. No such serious impairment of renal function developed in any of the human studies. In some of the animals, however, oliguria or anuria was produced by salt depletion and circulatory collapse, ureteral ligation, or bilateral nephrectomy (symbols with bars superimposed). In these, potassium remained in the cells. Hence, the ability or inability to excrete extracellular potassium is one factor which conditions movement of this cation out of cells.

On the other hand, entry of potassium into cells, in so far as it could be detected by the techniques employed in this study, appears to depend upon the availability of an exogenous supply of the cation. Without such a supply potassium entered cells in only 1 of the experiments recorded on Figure 1. Even this instance is questionable, however, since the amount, 30 m.eq., falls just outside the error inherent in the human experimental procedure. In sharp contrast to this finding, large amounts (100 to 200 m.eq.) entered the cells of each of the 3 patients given potassium (solid squares, Figure 1).

2. Transfers of sodium

In Figure 2 it can readily be seen that the wide variety of experimental procedures employed

³ This refers to the end result, and does not exclude the possibility that the process is initiated by alterations in the osmotic activity or the base-binding capacity of cellular anions.

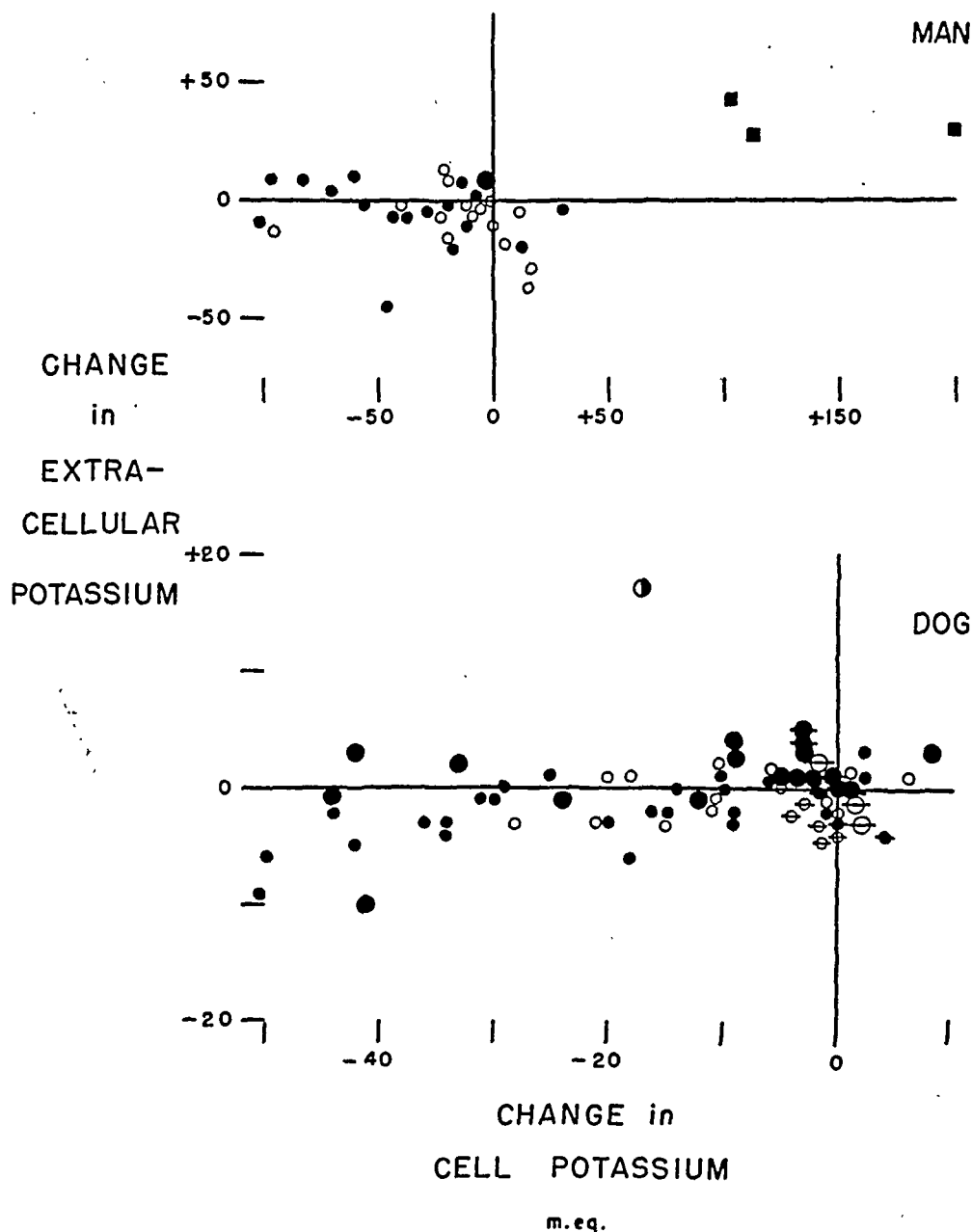


FIG. 1. RELATION CHANGES IN EXTRACELLULAR POTASSIUM TO CHANGES IN INTRACELLULAR POTASSIUM

Closed circles indicate experiments in which hypertonicity of body fluids was produced by the administration of hypertonic saline solutions (large closed circles), or by dehydration (small closed circles). Open circles represent studies in which body fluids became hypotonic as a result of the infusion of glucose solution into animals without renal function (large open circles), or as a result of salt depletion (small open circles). Superimposed bar on symbol identifies animals with oliguria or anuria. Closed squares represent patients given potassium. The half-closed circle indicates an animal in the agonal state.

tended to increase cell sodium, whereas under similar circumstances, as already seen in Figure 1, potassium usually declined. These cations differ in other respects as well. First, extensive increases of intracellular sodium did occur solely at the expense of extracellular sodium and inde-

pendently of an exogenous supply of the cation. Hypertonicity was the most frequent correlate of such a movement. Second, sodium left cells to enter the extracellular fluid in oliguric or in nephrectomized animals (symbols with superimposed bar). These observations contrast sharply

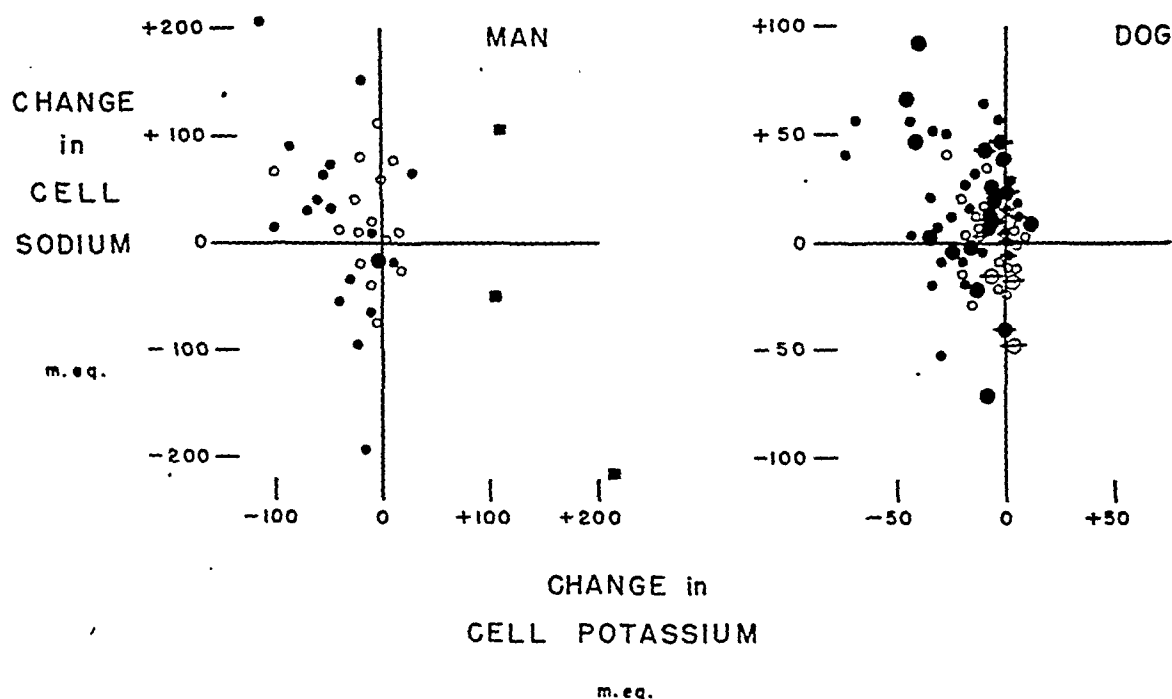


FIG. 2. RELATION OF TRANSFERS OF CELLULAR SODIUM TO THOSE OF CELLULAR POTASSIUM
Symbols are the same as in Figure 1.

with the behavior of potassium analyzed in the preceding section.

3. Relation of cell potassium to cell sodium transfers

There is evident no consistent quantitative reciprocal relationship between transfers of cell sodium and cell potassium (Figure 2). It can only be said that in a majority of the periods, and in 2 of the 3 cases in which potassium chloride was administered, the balance of cell potassium was negative and that of cell sodium was positive.

This was especially true in the dehydration experiments (small solid circles). The addition of sodium in excess of water (large solid circles) resulted in either a positive or negative balance of cell sodium, and only some of the positive balances were associated with losses of cell potassium. It is significant, however, that many animals in this category with increases in cell sodium without losses of cell potassium were anuric because of nephrectomy, ureteral ligation or salt depletion shock (symbols with superimposed bar). In these, therefore, the failure to observe reciprocal decreases or increases in potassium could be related

to the limitations imposed on potassium transfers in the absence of adequate renal function.

In the group of subjects who developed negative balances of cellular sodium, concomitant positive balances of cellular potassium, with but 2 exceptions (solid squares), were conspicuous by their absence. In those 2 experiments exogenous potassium was available. In the others it was not.

4. The relation of the net exchanges of cell cations to changes in osmotic activity (Figure 3)

There is a rough inverse correlation between the net exchanges of cell sodium and potassium and the internal alterations in osmotically active cell base, in that these 2 changes had the opposite signs in a majority of the periods. There was no quantitative relationship in most of the periods, however, and in many the changes were in the same direction. No one set of experimental conditions showed an increased correlation.

Experiments in which distortions of body water and electrolytes were partially or fully corrected sometimes showed reversal of these 2 values. In a total of 36 such periods, the net exchange of cell sodium plus potassium was reversed in 18, and

the balance change in osmotically active cell "base" was reversed in 20.

DISCUSSION

Certain generalizations concerning transfers of sodium and potassium are now permissible. It appears, for example, that large movements of potassium into cells can occur, provided that an exogenous supply of the cation is available. This has been observed in 1 patient given large amounts of carbohydrate as well as potassium chloride, and in 2 others who received potassium chloride in treatment of an attack of familial periodic paralysis. Apparently a similar penetration of exogenous potassium into cells can occur in the treatment of infant diarrhea and diabetic acidosis (4, 11). In these conditions, however, previous losses of potassium are being replaced. This may have been true in the 2 patients above with periodic paralysis. Such was definitely not the case in the patient given carbohydrate and potassium chloride.

It is not possible to state from data on hand whether an increase in the amount of potassium in an organism can *per se* lead to transfers of this ion into the cells. From *in vitro* blood cell studies it would appear that even a large concentration gradient in serum is not a sufficient impetus for the entry of potassium into cells, if metabolic processes are held in abeyance (24). Transfers of lesser magnitude are known to occur in association with metabolic activities without an exogenous supply of potassium. This has been observed *in vitro* during glycolysis in defibrinated blood, and in the pre-paralytic phase of periodic paralysis (8, 22, 25). Whether or not such small exchanges occurred in the *in vivo* experiments analyzed in this paper cannot be determined because they would fall in the range of the cumulative error of the analyses and calculations.

Movements of potassium out of cells, on the other hand, are, in part at least, dependent on adequate excretion of potassium. Cellular dehydration has been found to be the one disturbance most consistently conducive to the migration of potassium out of cells. However, in the absence of effective kidney function potassium during life does not leave its cellular position in any significant quantity, irrespective of any changes which may have been induced.

This dependency of potassium transfers out of

and into cells on an adequate renal function in the former instance, and on an exogenous supply of cation in the latter, contrasts sharply with the behavior of sodium which is subject to neither of these restrictions.

It is probable that reciprocal exchanges of these cations are modified by these restrictions which are imposed upon potassium transfers. As an inevitable corollary to these non-reciprocal movements of these 2 cations adjustment in behalf of osmotic forces must occur. This is accomplished in one or both of 2 ways. Water may traverse the cell membrane, or base in the cell may be inactivated or reactivated from the osmotic point of view.

Possible sources of error in the calculations which permit the above conclusions should be pointed out. The transfers of cell sodium and cell potassium are calculated on the assumption that the chloride in extracellular fluid does not enter cells and that chloride present in cells does not move out into the extracellular fluid. Obviously such transfers of chloride between cells and serum would affect the estimation of changes in extracellular volume from changes in the chloride space. If, for example, chloride did come out of the cells, false low values for the extracellular volume would be obtained. Conversely, if chloride entered cells, the extracellular volumes would be erroneously high. These deviations from actuality would in turn affect the estimation of sodium exchanges in particular, and to a minor and probably insignificant extent, those of potassium. It is possible, therefore, that in some of the experiments in Figures 1, 2, and 3 apparent transfers of sodium may merely represent unrecognized changes in the chloride space. It seems unlikely, however, that such transfers of chloride, if they did occur, could imperil the validity of the conclusions in this paper. First, it has been shown that measurements of extracellular volume by mannitol correlate closely with values obtained from alterations in the chloride space (22). Second, generalizations in this paper have been drawn only from experiments with changes in sodium and potassium definitely outside the possible sources of error, including those inherent in the use of the chloride space in calculating changes in extracellular water. Third, even though errors resulting from chloride transfers were present, they would, in general, merely

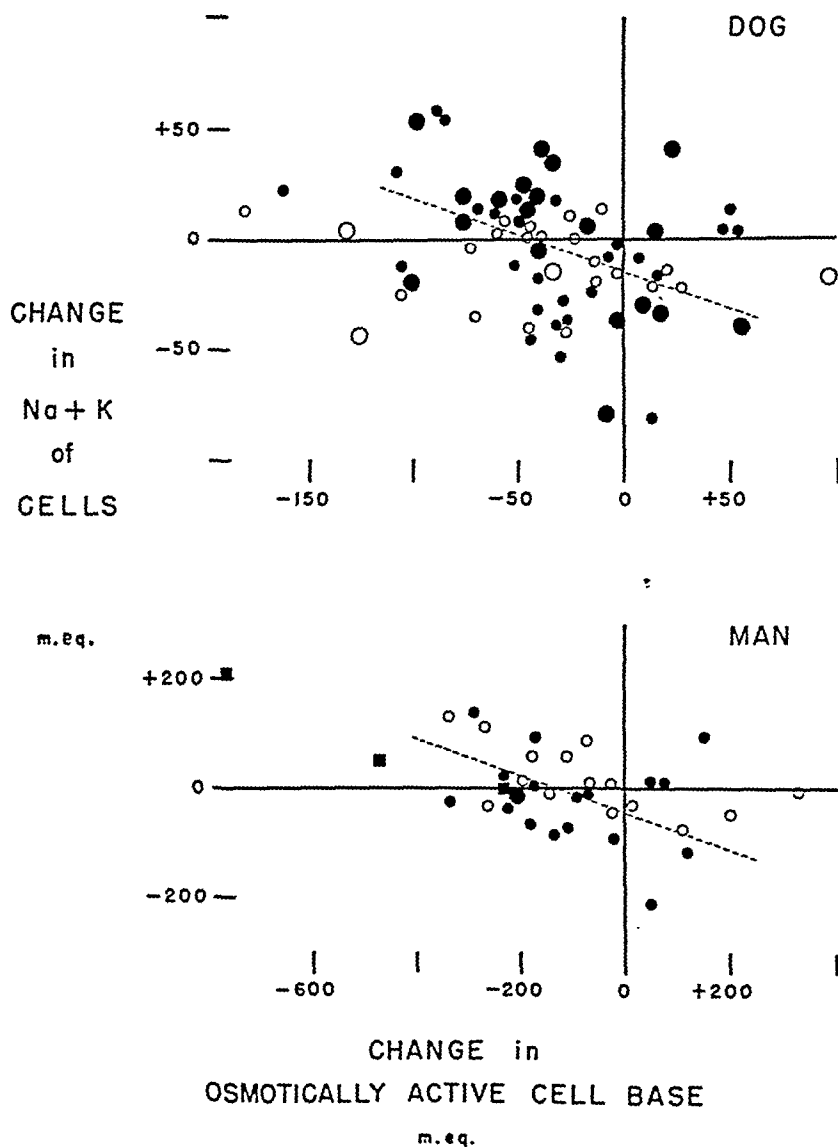


FIG. 3. RELATION OF THE SUM OF THE TRANSFERS OF CELLULAR SODIUM AND POTASSIUM TO CHANGES IN OSMOTICALLY ACTIVE CELL BASE

Symbols are the same as in Figure 1.

alter the degree rather than the direction of the transfers of cations. Finally, in any particular experiment, errors in calculating changes in extracellular volume would be common to both the sodium and potassium calculations. Hence the relations of these 2 transfers to each other would remain essentially unchanged. Actually, the chief error in the calculation of these transfers lies in the determination of serum sodium because the fraction in cells is such a small portion of the amount of sodium in the body.

Alterations in the chloride space do not enter

into the calculation of changes in osmotically active cell base. The sources of error in this calculation have been critically analyzed in an earlier paper (15). Concentration gradients between various compartments of the body fluids, for example, would invalidate some of the derived data. The duration of the experiments makes such gradients unlikely. Moreover, in many of the experiments the gradient would have to be opposite in direction to that expected from the conditions of the experiment, to account for the change in osmotically active base. Furthermore, even if an error of 4

m.eq. per liter (± 2) is allowed in the concentration of the serum sodium, it would not explain the discrepancies. The calculations of at least some of the larger changes in osmotically active base, therefore, are not invalidated by the above major sources of error.

The findings in these experiments permit discussion of certain ideas dealing with the distribution of cations between extracellular fluid and cells. Boyle and Conway (26) have propounded a theory which seemed to them to meet thermodynamic requirements; namely, that the muscle cell membrane is impermeable to extracellular sodium and intracellular anions (phosphate and protein). Therefore, potassium must be intracellular and chloride and bicarbonate extracellular. If a small amount of chloride and bicarbonate were intracellular, an equivalent amount of potassium would be extracellular. On this model increments or decrements of potassium would be distributed through both phases in proportion to the volume of water in each phase. The theory fails, however, to explain the exchange of sodium for potassium in the cell and it ignores the relation of transfers of K to oxidative reactions (25). Figure 1 clearly indicates that potassium is not added to or subtracted from the 2 phases of body fluid in proportion to their relative volumes, as the theory of Boyle and Conway would require. The ratio of the increments or decrements of cell potassium to those of extracellular potassium was frequently of much greater magnitude than that of the ratio of intracellular to extracellular volume. In other words considerable amounts of cell potassium were transferred across the extracellular phase with very little change in potassium content of the latter. Other mechanisms than isotonic distribution of potassium between the 2 phases must be responsible. Furthermore, the lack of a close relationship between transfers of cell sodium and cell potassium suggests at least semi-independent control of each ion. In Figure 2 it is seen that there is no strictly quantitative reciprocal relation between the two in a great majority of the experiments. These findings are consistent with the data of Darrow (11) which demonstrated inverse balances of cell sodium in only half of the cases of infant diarrhea with depletion of cell potassium. The evidence does not support the concept that cellular sodium is merely a substitute for missing cell

potassium. The data presented in this paper support the theory that cation exchanges across the cell boundary are related to metabolic activities of the cell and the expenditure of energy, rather than to passive transfers resulting from differential impermeabilities.

The factors which alter the osmotic activity of the cellular components cannot be defined from the experiments at hand. The most striking fact about these alterations is their magnitude. As indicated earlier the balance of osmotically active cell base greatly exceeds that of the net exchanges of sodium and potassium across the cell boundary. This fact suggests that this reaction may sometimes be primary, and not secondary to the net transfers of cations into or out of the cells. Certainly from the standpoint of shifts of water, a change in the osmotic activity of cell components is often the most important determinant of the ultimate distribution of water between the phases of body fluid. The most striking example of this fact is present in dog 115 (20) in which at 2.7 hours *all* of the 1.1 liter of water given as 5 per cent glucose solution was retained in the extracellular phase *plus* an additional 0.1 liter transferred from the intracellular phase. Short of a very large and unlikely water gradient persisting between the 2 phases, this peculiar distribution of water can only be explained by the inactivation of a large amount of cell base, or by a complete failure of glucose to diffuse across the cell membrane. Similarly in prolonged water deprivation, the amount of water released from the intracellular phase by the inactivation of base greatly exceeds that removed by net transfers of cations out of the cell, by breakdown of protein, and by osmotic shifts (16). Whether or not such inactivation, or reactivation, serves a useful purpose is not known.

SUMMARY AND CONCLUSIONS

1. Potassium leaves cells in significant amounts only if it can be excreted. Hypertonicity and cellular dehydration are frequent, though not invariable, correlates of such movements.
2. Transfers of large amounts of potassium into cells have been observed only when exogenous potassium was available.
3. Sodium, unlike potassium, can enter the serum from cells even though renal function is greatly impaired or ceases entirely.

4. In contrast to potassium large increases in cell sodium are not dependent upon the exogenous supply of this cation.

5. Exchanges of sodium for potassium in cells do occur. This is not, however, an invariable finding in any particular experimental procedure, nor are the transfers, when present, always of equivalent magnitude.

6. Alterations in the osmotic activity of cations of cells are not correlated closely with either the direction or the magnitude of the transfers of sodium or of potassium.

7. The implications of these data with respect to the theories concerning the distribution of cations between cells and extracellular fluid are discussed.

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STUDIES IN SERUM ELECTROLYTES. XV. THE CALCIUM-BINDING PROPERTY OF THE SERUM PROTEINS

(MULTIPLE MYELOMA, LYMPHOGRANULOMA VENEREUM AND SARCOIDOSIS)

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Rona and Takahashi (1) in 1911 first demonstrated by dialysis experiments that serum calcium exists in 2 forms: diffusible, and nondiffusible. Later work by Cushing (2) and by Neuhausen and Pincus (3) employing pressure filtration through collodion membranes demonstrated that approximately half of the total serum calcium is present in either form. Subsequent investigators have confirmed these findings (4) and have shown that in man most of the diffusible calcium is ionized, and most of the nondiffusible calcium is bound to the serum proteins (5).

Previous measurements of the relative amounts of calcium bound by the albumin and globulin fractions of the total serum protein have been unsatisfactory, owing to the difficulty encountered in separating and purifying the proteins without altering their calcium-binding property. As late as 1942 Masket, Chanutin, and Ludewig (6) indicated that the calcium-binding properties of serum albumin and globulin as determined by various investigators were not in agreement presumably owing to differences in the techniques employed. The earlier methods of protein separation were dependent upon salting out procedures which, because of the excess of salts present, required subsequent dialysis in order to purify the proteins. It would seem reasonable that such manipulations might alter the calcium-binding power. In addition, it has since been amply demonstrated that the salting out procedures do not give an albumin-globulin separation comparable to that of electrophoresis (7, 8).

Csapo and Faubl (9) observed that less calcium is carried down by the globulin fraction precipitated with half-saturated ammonium sulfate than by the albumin fraction. Bendien and Snapper (10), likewise, on the basis of protein separations performed by precipitating the globulins with sodium sulfate concluded that most of the non-

diffusible calcium is bound to the albumin fraction. This view is also held by Schmidt and Greenberg (11). McLean and Hastings (5) on the basis of measurements made on purified horse serum proteins concluded that the values of the calcium-binding property are 0.716 mgm. of calcium per gram of albumin and 0.744 mgm. of calcium per gram of globulin. Drinker, Green, and Hastings (12), on the basis of measurements on purified fractions of horse serum globulin, concluded that the value of the calcium-binding property varies from 0.20 to 3.44 mgm. of calcium per gram of globulin.

Values for the calcium-binding property of protein have been estimated statistically by plotting the concentrations of the total serum calcium against the concentrations of total serum protein in a scattergram, and applying the formula of Hastings, Murray, and Sendroy (13):

$$[\text{Total Calcium}] = m [\text{Total Protein}] + b$$

where "b" represents the diffusible calcium in mgm. per 100 ml. (assuming the latter to be constant) and "m" represents the average binding property of the serum proteins in milligrams per gram of protein. By this method Hastings, Murray, and Sendroy conclude that the value of $m = 0.56$, and that of $b = 5.6$. Peters and Eiserson (14) report the values to be $m = 0.56$, $b = 6.0$; and Greenwald (15) observes that $m = 0.875$, and b varies from 3.7 to 5.0 in the adult and is 6.3 in infants.

This statistical approach by the scattergram method has been applied by Gutman and Gutman (16) to the study of sera obtained from patients suffering from lymphogranuloma venereum and other chronic diseases which present elevated concentrations of serum globulin. Cases of multiple myeloma were not included in this study since the view was held that the increase in serum calcium frequently observed in this condition was

often due to an increase in the diffusible fraction. It was found that when the serum globulin rose above 4.0 grams per 100 ml., the deviation from the Hastings formula was roughly proportional to the increased concentration of globulin. It was calculated that the excess globulin bound only 0.1 to 0.2 mgm. of calcium per gram of excess globulin. As this amount was considered to be negligible, the following values were introduced into the Hastings formula:

$$[\text{Total Calcium}] = 0.83 [\text{Albumin}] + 7.0.$$

The value of 0.83 represents the calcium-binding property of albumin in mgm. of calcium per gram of albumin. The value of 7.0 is a constant and represents the diffusible calcium plus the calcium bound to globulin.

In 1944 Pillemer and Hutchinson (8) reported a method for the separation of albumin and globulin which gave values that compared favorably with those obtained by electrophoresis. In addition, the method avoided the use of saturated salt solutions and thus appeared to be suitable for the measurement of the calcium bound to each protein fraction. As the normal calcium-binding property of the individual serum proteins, in the opinion of most investigators, has never been determined in a satisfactory manner the following experiments were undertaken to attempt the estimation of these values, and to study their variations in certain diseases.

METHODS

Diffusible calcium was estimated by measuring the concentration of calcium in a protein-free ultrafiltrate of serum. The amount of calcium bound to globulin was estimated from the difference between the concentration of calcium in the original serum, and the concentration of calcium in the same serum after precipitation of the globulins with methanol. The remaining calcium fraction was considered to be bound to albumin.

Samples of blood were withdrawn under oil without stasis and allowed to clot in the refrigerator. The sera were separated approximately 2 hours after withdrawing the blood, and the analyses were made within 48 hours.

Protein-free filtrates were prepared by suction filtration through "Nojax" casing¹ membranes using a Greenberg-Gunther (17) apparatus modified to provide an extra trap and a more reliable method for maintaining water-vapor saturation within the tube containing the filtrate (Figure 1). No protein was demonstrable in the ultrafiltrates by the biuret reaction. The values for specific gravity of the ultrafiltrates ranged from 1.007 to 1.008.

For purposes of comparison, the concentration of calcium and protein have been expressed in terms of serum water. The specific gravity of sera and ultrafiltrates was measured by means of a 2-ml. pycnometer. The percentages of total solids in sera and in ultrafiltrates were obtained by drying weighed samples at 100° to 105° C. to constant weight. The concentration of calcium and protein in relation to the concentration of water was calculated from these measurements in accordance with Sunderman's method (18) for the calculation of such factors.

Measurements of the total serum protein and the protein fractions were made by a modification of the biuret

¹ Made by the Visking Corp., Chicago, Illinois. Diameter 23/32".

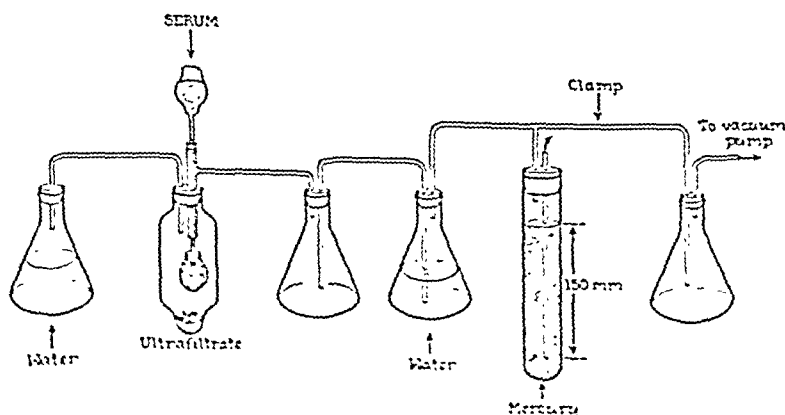


FIG. 1. MODIFIED GREENBERG-GUNTHER APPARATUS FOR THE PREPARATION OF PROTEIN-FREE ULTRAFILTRATES

Serum is contained in semipermeable membrane, and constituents of the ultrafiltrate are drawn through by means of negative pressure. Note water flasks for maintaining constant water vapor pressure within system.

TABLE I

Original data: Measurement of calcium and protein fractions in normals, multiple myeloma, sarcoid, and lymphogranuloma venereum

Diagnosis	Case	Total calcium serum	Diffusible calcium filtrate	Calcium in globulin-free serum	Total protein serum	Albumin (methanol method) serum	Albumin (salting method) serum	Specific gravity 20/20	Per cent solids serum
		mgm./100 ml.	mgm./100 ml.	mgm./100 ml.	grams/100 ml.	grams/100 ml.	grams/100 ml.	°C.	grams/100 grams
Normals	DL	10.42	4.76	8.09	6.67	4.23	4.87	1.0271	8.64
	AR	10.36	4.16	8.00	6.83	4.00	4.97	1.0261	8.51
	JM	10.20	4.99	7.84	7.13	4.09	4.79	1.0272	8.66
	FS	10.00	4.84	8.09	6.83	4.26	5.00	1.0266	8.52
	AP	10.00	4.99	7.28	6.70	3.40	4.38	1.0274	8.39
Multiple myeloma	JS	16.93	4.12	8.53	10.57	0.79	1.31	1.0452	14.08
	NA	12.20	4.58	7.52	8.05	1.22	2.78	1.0340	11.22
	LP	11.10	4.37	8.73	5.62	3.73	4.22	1.0245	7.63
	LT	11.00	5.72	7.60	5.58	2.15	3.22	1.0253	7.60
	JM	10.80	4.78	8.73	6.21	2.94	4.69	1.0271	8.29
	ES	9.17	4.88	6.32	4.62	1.88	3.14	1.0228	6.65
Sarcoid	JP	10.21	4.65	6.45	7.26	1.68	2.52	1.0289	9.76
Lympho-granuloma venereum	MC	10.50	4.37	9.00	7.16	2.44	3.83		
	TB	9.70	3.54	6.55	7.72	2.80	4.10	1.0318	9.90
	HF	8.88	4.10	5.79	7.72	0.56	0.80	1.0306	9.74
Healed Lympho-gran. ven.	JL	10.21	5.20	7.64	6.76	3.17	3.58	1.0265	8.97

method (19) and the Pregl method for nitrogen (20). The albumin and globulin fractions were separated by the method of Pillemer and Hutchinson (8), in which the globulins are precipitated by methanol and separated by filtration at a temperature below 10° C. The presence of small amounts of methanol was shown not to affect the biuret reaction.

Estimation of the albumin and globulin fractions was also obtained by salting out the globulin with Na_2SO_4 according to the method of Howe (21) as modified by Kingsley (22).

The concentrations of calcium in solution were estimated by the Kramer-Tisdall procedure as modified by Tisdall (23) and by Clark and Collip (24). This procedure was found to yield satisfactory values in serum, in protein-free ultrafiltrates, and in methanol filtrates obtained with the method of Pillemer and Hutchinson. The amount of soluble calcium oxalate, which was found to be negligible in the methanol solutions used, was taken into account in the calculations. In calculating the concentration of calcium in the globulin-free serum filtrate the volume previously occupied by the globulin was neglected since it was shown to amount only to about 7 parts per 1000 parts of filtrate.

All measurements of protein and calcium were performed in duplicate. When the duplicate measurements did not agree within 4 per cent of each other, they were repeated.

RESULTS

The original data from which the calculations have been made are presented in Table I.

In Table II are given the values for the concentrations of the various calcium and protein fractions, calculated in relation to the concentrations of water. The calculated calcium-binding properties of the protein fractions are also shown.

Comparisons of albumin-globulin ratios obtained by methanol precipitation, salting out, and by electrophoresis are given in Table III. It will be seen that our estimations of the albumin-globulin ratios obtained from methanol precipitation of globulin agree reasonably well with those obtained by electrophoresis and thus would confirm the findings of Pillemer and Hutchinson. It has been shown by Dole and Braun (7) that in normal sera the value of the A/G ratio obtained by electrophoresis bears a relation to the value of the A/G ratio obtained by the salting out method of approximately 2:3. A similar relationship between the values of the A/G ratios obtained by methanol precipitation and salting out, respectively, was observed in our normal group.

DISCUSSION

The results obtained in this study are based upon the assumption that globulin does not lose its bound calcium as a result of precipitation of

TABLE II

Calculated data: Calcium and protein partition, and calcium-binding power of the serum proteins in normal and abnormal conditions

Diagnosis	Case	Calcium partition					Protein partition *			Binding power		Increased globulin fraction
		Total	Diffusible	Bound	Bound to albumin	Bound to globulin	Total	Albumin	Globulin	Albumin	Globulin	
		mgm. per 100 grams H ₂ O	mgm. per 100 grams H ₂ O	mgm. per 100 grams H ₂ O	mgm. per 100 grams H ₂ O	mgm. per 100 grams H ₂ O	grams per 100 grams H ₂ O	grams per 100 grams H ₂ O	grams per 100 grams H ₂ O	mgm. per gram	mgm. per gram	
Normals	AR	11.03	4.18	6.85	4.34	2.51	7.27	4.26	3.01	1.02	0.83	
	DL	11.11	4.78	6.33	3.84	2.49	7.11	4.51	2.60	0.85	0.96	
	FS	10.65	4.86	5.79	3.75	2.04	7.27	4.54	2.73	0.83	0.75	
	JM	10.87	5.01	5.86	3.34	2.52	7.60	4.36	3.24	0.77	0.78	
	AP	10.62	5.01	5.61	2.71	2.90	7.12	3.61	3.51	0.77	0.83	
Average										0.85 (s.e. = 0.045)	0.83 (s.e. = 0.040)	
Multiple myeloma	JS	18.86	4.14	14.72	5.38	9.34	11.77	0.88	10.89	6.11	0.86	β
	NA	13.29	4.60	8.69	3.59	5.10	8.77	1.33	7.44	2.70	0.68	γ
	JM	11.47	4.80	6.67	4.47	2.20	6.60	3.12	3.48	1.43	0.63	γ
	LP	11.73	4.39	7.34	4.83	2.51	5.94	3.94	2.00	1.23	1.26	α
	LT	11.62	5.74	5.88	2.29	3.59	5.89	2.27	3.62	1.01	0.99	α
	ES	9.60	4.90	4.70	1.73	2.97	4.84	1.97	2.87	0.88	1.03	α
Sarcoid	JP	11.00	4.67	6.33	2.28	4.05	7.82	1.81	6.01	1.26	0.67	γ
Lympho-granuloma venereum	HF	9.55	4.12	5.43	2.11	3.32	8.30	0.60	7.70	3.52	0.43	γ
	MC	11.19	4.39	6.80	5.20	1.60	7.63	2.60	5.03	2.00	0.32	γ
	TB	10.44	3.56	6.88	3.48	3.40	8.31	3.01	5.30	1.16	0.64	γ
Healed lymphogran. venereum	JL	10.92	5.22	5.70	2.96	2.74	7.23	3.39	3.84	0.87	0.71	

* By the method of Pillemer and Hutchinson (8).

TABLE III

*Comparison of methanol, * Na₂SO₄,† and electrophoretic separation of albumin and globulin*

Diagnosis	Case	Albumin		Globulin		A/G ratio		
		Methanol	Na ₂ SO ₄	Methanol	Na ₂ SO ₄	Methanol	Na ₂ SO ₄	Electrophoresis
		grams/100 grams H ₂ O	grams/100 grams H ₂ O	grams/100 grams H ₂ O	grams/100 grams H ₂ O			
Normals	AR	4.26	5.29	3.01	1.98	1.42	2.67	
	JM	4.36	5.11	3.24	2.49	1.34	2.05	
	FS	4.54	5.32	2.73	1.95	1.66	2.73	
	DL	4.51	5.19	2.60	1.92	1.73	2.70	
	AP	3.61	4.65	3.51	2.47	1.03	1.88	
Multiple myeloma	ES	1.97	3.29	2.87	1.55	0.69	2.12	0.62
	LT	2.27	3.40	3.62	2.49	0.63	1.36	0.46
	LP	3.94	4.46	2.00	1.48	1.97	3.01	1.30
	JS	0.88	1.46	10.89	10.31	0.08	0.14	0.23
	NA	1.33	3.03	7.44	5.74	0.18	0.53	0.26
	JM	3.12	4.98	3.48	1.62	0.90	3.07	1.00
Sarcoid	JP	1.81	2.71	6.01	5.11	0.30	0.53	
Lympho-granuloma venereum	HF	0.60	0.86	7.70	7.44	0.07	0.11	
	MC	2.60	4.08	5.03	3.55	0.52	1.15	
	TB	3.01	4.41	5.30	3.90	0.57	1.13	
Healed lymphogran. venereum	JL	3.39	3.83	3.84	3.40	0.88	1.13	

* By the method of Pillemer and Hutchinson (8).

† By the method of Howe (21).

the protein. To our knowledge, no evidence bearing upon this assumption is available.

McLean and Hastings (5) and others (6, 25) have indicated that in solutions containing calcium and protein, the following mass law relationship holds as a first approximation:

$$I. \quad \frac{[\text{Calcium Proteinate}]}{[\text{Ca}^{++}] \times [\text{Protein}^-]} = K.$$

From this equation the amount of calcium bound to protein would be partially dependent upon the concentration of calcium ion. Comparisons are, therefore, made only upon those cases having a diffusible calcium concentration within a relatively narrow range (4.18 to 5.22 mgm. per 100 grams H_2O). One case of lymphogranuloma (TB) had a diffusible calcium concentration below this range, and 1 case of multiple myeloma (LT) had a concentration above it. The calculated binding power of the proteins in these cases is not considered to be comparable with others of the series.

The average value for the calcium-binding property of total serum protein in our normal subjects was 0.84 (s.e. = 0.029) mgm. of calcium per gram of protein. This value compares favorably with that of 0.87 derived statistically by Greenwald (15). It is, however, higher than that of 0.56 by Hasting *et al.* (13) and by Peters and Eiserson (14). The average value for the calcium-binding property of serum globulin in our series was 0.83 (s.e. = 0.040) mgm. of calcium per gram of total globulin which is fairly close to the value of 0.74 obtained by McLean and Hastings (5) for horse serum globulin. The average calcium-binding property of albumin from normal subjects was 0.85 (s.e. = 0.045) mgm. per gram, as compared to the value of 0.716 obtained by McLean and Hastings (5). These authors indicate that owing to the tendency of serum albumin to lose its calcium-binding power during purification, it would seem likely that their value did not represent the full calcium-binding power of serum albumin under natural conditions.

It has long been known that the concentration of serum albumin shows a strong tendency to decrease in diseases characterized by hyperglobulinemia. Heretofore, studies of the properties of albumin in hyperglobulinemic sera by various precipitation procedures (26, 27) and by electrophoresis (28 to 32) have shown no indication of

any qualitative change in the albumin fraction. In the experimental results presented in this paper, it will be noted that most of the patients studied show an increase in the calcium-binding property of their serum albumin, some to a marked degree. This phenomenon is seen in all 3 diseases.

If it be assumed that

$$[\text{Ca Proteinate}] = [\text{Total Calcium}] - [\text{Ca}^{++}]$$

following the relationships of Marrack and Thacker (33) and McLean and Hastings (5), then from Equation I:

$$II. \quad \frac{[\text{Total Calcium}] - [\text{Ca}^{++}]}{[\text{Ca}^{++}]} = K [\text{Total Protein}].$$

McLean and Hastings (5) have shown that in human serum practically all the diffusible calcium is ionized, and the non-diffusible calcium is practically all bound to protein. If, therefore, on the left-hand side of the Equation II, the expression [Calcium bound to Protein] is substituted for the numerator, and the expression [Diffusible Calcium] is substituted for the denominator, then:

$$III. \quad \frac{[\text{Calcium bound to Protein}]}{[\text{Total Protein}]} = K [\text{Diffusible Calcium}].$$

If we now consider the specific application of Equation III to albumin:

$$IV. \quad \frac{[\text{Calcium bound to Albumin}]}{[\text{Albumin}]} = K [\text{Diffusible Calcium}].$$

Equation IV is plotted in Figure 2, "K" having been determined from the data for normal sera. The points obtained from normal sera are all close to the curve. The points derived from the sera obtained from patients, however, are for the most part well outside the range of dispersion of the normals. This suggests either that an appreciable amount of non-diffusible calcium is bound to substance other than protein, or else that a protein different from normal serum albumin makes its appearance in the albumin fraction in significant quantity in the diseases studied.

The calcium-binding property of globulin in the sera from patients with multiple myeloma was increased above the normal range in 3

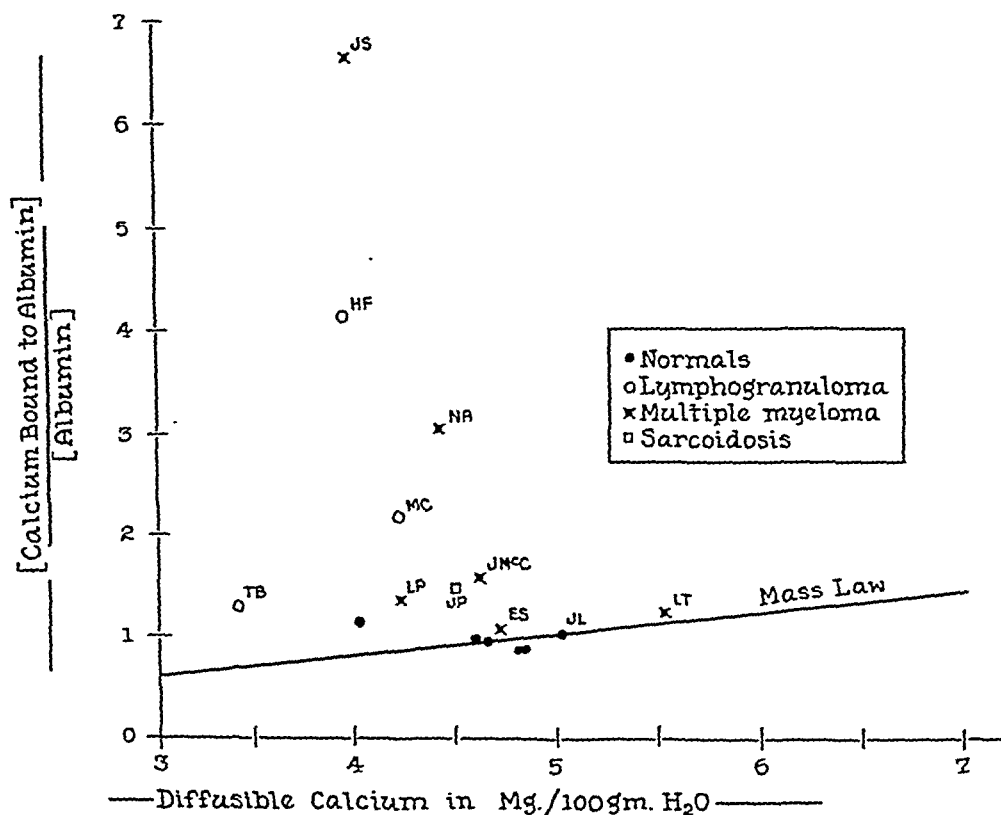


FIG. 2. EQUATION IV PLOTTED FOR ALBUMIN OF NORMALS AND OF CASES OF MULTIPLE MYELOMA, SARCOIDOSIS, AND LYMPHOGRANULOMA VENEREUM

sera, normal in 1, and below normal in 2. Variation might be expected on the basis of electrophoretic patterns (34 to 37) and of studies of the base-binding properties of globulin (16, 38, 39). In the cases characterized by a predominance of the alpha globulin fraction, the average calcium-binding property of globulin is elevated. Where the beta fraction predominates, the average calcium-binding property is normal. As surmised by Gutman and Gutman (16), gamma globulin appears to have a low calcium-combining power. The sera from patients with lymphogranuloma venereum, sarcoidosis, and tuberculosis have characteristically an increase in serum gamma globulin (29, 30, 32, 40, 41). In the 2 long-standing cases of lymphogranuloma venereum in our series, HF and MC, a marked decrease was apparent in the calcium-binding property of serum globulin. Values at the lower end of the normal range were found in the early cases of lymphogranuloma venereum and in the case of sarcoidosis. According to the data of West and Jefferson

(42), the total calcium tends to decrease in tuberculosis, and it is likely that the relative increase in gamma globulin found in this disease may be the cause of this.

The considerable differences in the calcium-binding power of the alpha, beta, and gamma globulin fractions suggested by our data are in harmony with the findings of Drinker, Green, and Hastings (12). It should be noted in this connection, however, that the actual proteins in the various globulin fractions separated by electrophoresis, may differ in disease from those found in the normal, for as shown by Cohn (43), each fraction probably consists of a mixture of proteins of similar physical characteristics.

The mechanism of hypercalcemia in multiple myeloma, has long been a controversial point. Cantarow (44) pointed out that, although plausible, the hypothesis that hypercalcemia in multiple myeloma is dependent upon hyperproteinemia, is far from being well-established. The fact that there often was no correlation between the con-

centration of total serum calcium and total serum protein in this disease convinced some authors that the mechanism was not one primarily dependent upon the calcium-protein relationship (26, 27, 45). It has been shown by Gutman and Gutman (26) that in some cases the hypercalcemia is caused by an increase in the diffusible calcium fraction, presumably owing to breakdown of bone. Case LT in our series illustrates this condition. The other cases in the present series, however, indicate that in some instances, at least, the hypercalcemia is directly dependent upon alteration of the calcium-binding property of serum protein. The combination of increased binding property of the serum albumin and increased concentration of serum globulin having normal or elevated binding power, may lead to an increase in bound calcium. Hypercalcemia is not observed in such diseases as lymphogranuloma venereum presumably owing to the low calcium-binding power of the gamma globulin formed in this disease.

SUMMARY

The calcium-binding property of serum albumin and globulin obtained from normal subjects has been estimated. It was found that approximately the same amount of calcium is bound by both albumin and globulin, the average being 0.84 mgm. of calcium per gram of protein.

In diseases characterized by abnormality of the serum proteins, the calcium-binding property of albumin may increase, in some cases to as much as 6 times the normal value. The calcium-binding property of globulin in such diseases varies from the normal, and the direction of variation appears to depend upon the predominating electrophoretic globulin fraction present.

One or more of several mechanisms appear to be responsible for the hypercalcemia frequently observed in multiple myeloma. These include increased calcium-binding property of the serum albumin; increased concentration of serum globulin; and increased concentration of diffusible calcium.

Dr. Florence B. Seibert made the electrophoretic measurements which the authors gratefully acknowledge.

CLINICAL ABSTRACTS

1. JS (Lankenau Hospital No. A47636). Patient was a 56 year old married negro male who had bone pains, weakness and weight loss for 8 months, and recurrent

epistaxis for 1 month. Physical examination was essentially unrevealing except for emaciation. X-ray films showed numerous areas of bone destruction in the skull, ribs, pelvis, and the lumbar vertebrae. A mass projected from a destroyed rib into the thoracic cage. Examination of the sternal marrow revealed typical myeloma cells. Urine contained Bence-Jones protein, and a 24-hour specimen contained 3.2 grams of albumin per liter. The erythrocyte count was 3.5 million per cmm. The leucocyte count was 14,100 per cmm., with neutrophils 72 per cent, and lymphocytes 28 per cent. Serum inorganic phosphorus was 5.0 mgm. per 100 ml. The blood clotted rather slowly, but once clotted formed a firm mass from which serum was not expressed.

2. LT (Pennsylvania Hospital No. 77922), a 67 year old white male admitted with a fracture of the right humerus. Patient had a plasmacytoma removed from the right nostril 8 years ago. Numerous recurrences were treated with further surgery and with irradiation. Two years ago he developed collapse of the fourth and fifth lumbar vertebrae, and 1 year ago he developed a fracture of the right clavicle. A course of stilbamidine was given. Temperature on admission was 102° F. There was crusting and pungent odor in the right nostril. There was a mass in the right clavicle, and deformity of the right humerus. X-rays revealed multiple lesions in the right humerus, radius and scapula, in the ribs, skull, and the left femur.

3. LP (U. of P. Hospital No. 4781183), a 71 year old colored man, who in August, 1946, noted a lump over the sternal region. There was slow enlargement of the lump with pain in the region. There was some cough and wheezy respiration. In December, 1946, patient developed swollen abdomen and edema of the legs. On physical examination there was some mottled pigmentation of the legs. A hard rounded walnut-sized tumor was present over the left fourth costochondral junction. Rhonchi were heard throughout the chest. There was a systolic murmur, but no cardiac enlargement. The liver was felt 2 fingers below the costal margin. Pitting edema of the legs was present. Biopsy of the sternal mass revealed plasma cell myeloma. X-ray studies revealed multiple bony defects in the skull, humeri, scapulae, clavicles, sternum, and thoracic cage. There seemed to be a mass behind the heart. Leucocyte count was 12,350 per cmm. with 20 per cent plasmocytes in the peripheral blood. Urine showed moderate albumin, and contained Bence-Jones protein. There was a progressively increasing anemia.

4. NA (U. of P. Hospital No. 4467961). Patient was a 55 year old white female who complained of low back pain, left knee pain, and increasing fatigue for 1 year. Physical examination was essentially negative. X-ray films revealed punched-out areas of bone destruction in the skull, ribs and long bones. Pathologic fractures were sustained above the left knee, and subsequently in the other femur and a humerus. Sternal marrow and rib biopsy both showed typical myeloma cells. Urea clearance was 47 per cent of normal. Urine never contained Bence-Jones protein, but showed 1+ to 2+ albumin.

Leucocyte cell count ranged from 2300 to 5500 cells per cmm. Erythrocyte count ranged from 2.4 to 2.9 million per cmm. Treatment with radioactive phosphorus, X-rays, and intravenous stilbamidine was attempted without noteworthy result.

5. JM (Temple U. Hospital No. 122318). Patient was a 52 year old white male who complained of weakness and tiredness for 1 year, following an attack of "pleurisy." There was a 50-pound weight-loss. Physical examination revealed a large liver and spleen. X-ray films of the bones were negative. Chest X-ray films revealed some rounded soft densities, the nature of which was uncertain, in the right lung, as well as some pneumonitis. Examination of the sternal marrow showed typical myeloma cells. Urine contained no Bence-Jones protein. Leucocyte count ranged from 3400 to 5400 per cmm., neutrophils 66 to 73 per cent, lymphocytes 24 to 25 per cent, monocytes 3 to 8 per cent. Autopsy showed widespread myelomatosis.

6. ES (Temple U. Hospital No. 122883). Patient was a 58 year old white male who complained of weakness of the legs for 2 months, and inability to stand for 3 weeks. Physical examination suggested a tumor at the fourth thoracic vertebra with signs of pressure on the cord at this level. X-ray films showed complete destruction of the body of this vertebra with kyphosis. The tumor was removed and proved to be plasma cell myeloma. There was no evidence of other lesions. Urine contained no Bence-Jones protein.

7. HF (Philadelphia General Hospital No. 170882). Patient was a 48 year old negro female, who was found to have a rectal stricture 1 year before performance of the present studies. The lygranum test was moderately positive at the time the stricture was first noted. A colostomy was performed because of progressive narrowing of the rectal lumen.

8. MC (U. of P., O.P.D. No. 43176). Patient was a 31 year old married negro female, who gave a history of rectal stricture and bleeding of 3 years' duration. Physical examination was unrevealing except for the presence of a stricture 1 inch above rectal sphincter. The lygranum test, done shortly before the present studies, was strongly positive.

9. TB (U. of P., O.P.D. No. 42189). Patient was a 25 year old married negro male, who noted bilateral inguinal tenderness and swelling 3 weeks prior to the present studies. The inguinal lesions progressed to typical buboes. Aspiration of these revealed frank pus. Oral temperature was 100.0° F. A lygranum test was applied, but the patient did not return for follow-up.

10. JL (U. of P. Hospital No. 4680289). Patient was a 28 year old married negro male, who noted 3 shallow penile ulcers, and right inguinal tenderness. The latter progressed to a typical bubo, and yielded frank pus on aspiration. The lygranum test was positive. Two weeks after the onset of his illness, serum protein was 6.8 per cent, albumin 2.8, globulin 4.0 (by method of Howe). Sulfadiazine therapy was started at this time, and continued for 1 month at which time the present studies were

performed. At this time, symptoms and signs of the disease had completely subsided.

11. JP (U. of P. Hospital No. 4677177). Patient was a 13 year old negro male, who over an 8-month period had had enlarged tender parotids, swelling of the upper lids, night sweats, dyspnea, tonsillitis, and cervical adenitis. X-ray films of the chest were reported as showing "numerous areas suggesting interstitial infiltration almost approaching nodulation." Tuberculin tests were consistently negative. Biopsy of a cervical lymph node showed tubercles composed of macrophages and multinucleated giant cells without caseation. The histologic appearance was considered to be typical of sarcoid.

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SERUM PRECIPITABLE IODINE CONCENTRATIONS DURING PREGNANCY¹

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Circulating thyroid hormone is measured more accurately by determination of serum precipitable iodine than of basal metabolic rate (1 to 8). Since the latter increases during pregnancy (9 to 14) serum precipitable iodines were investigated in 43 pregnant women and followed in some subjects after delivery.

¹A preliminary report of this investigation was included in the Proceedings of the Thirty-Eighth Annual Meeting of the American Society for Clinical Investigation, J. of Clin. Invest., 1946, 25, 926.

SUBJECTS AND METHODS

The ages of the 43 subjects ranged from 21 to 44 years. Twenty-nine subjects were normal; they had neither family nor personal histories suggestive of metabolic disturbances. Physical examinations did not reveal abnormalities in pulse rate, pulse pressure, size and consistency of the thyroid gland, tremor or eye signs. Fifteen drops daily of Lugol's solution were given to 2 of the subjects for 5 and 9 weeks, respectively. One subject received 100,000 units of estradiol in oil intramuscularly within 1 week. Of the remaining 14 women, 4 had hyperthyroidism, 4 miscarried, and 6 who did not miscarry were given

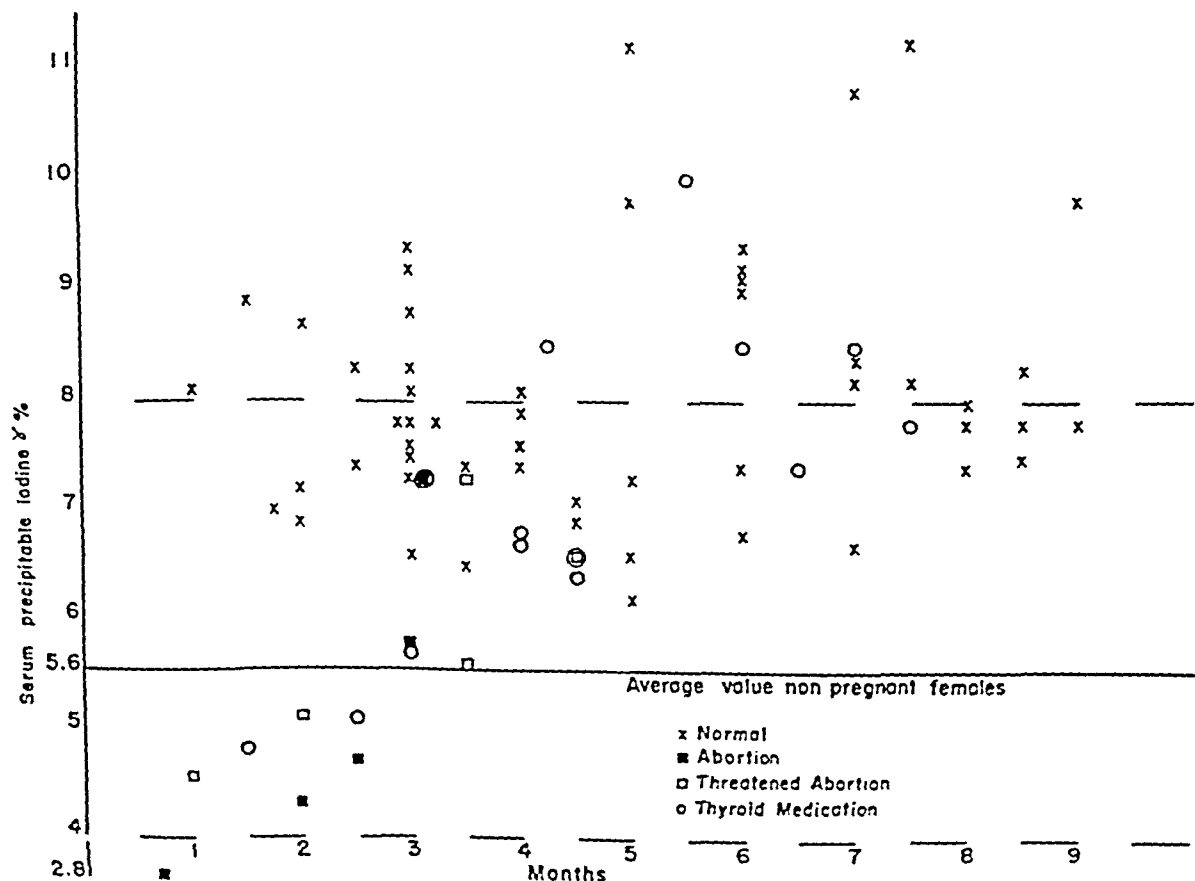


FIG. 1. SERUM PRECIPITABLE IODINES OF 39 PREGNANT WOMEN, EXCLUSIVE OF PATIENTS WITH HYPERTHYROIDISM

Broken horizontal lines represent the upper and lower limits of normal range of serum precipitable iodine (4.0 to 8.0 gamma per cent). Solid horizontal line is 5.6 gamma per cent, the average normal value from statistical analysis (16).

desiccated thyroid although the diagnosis of hypothyroidism could be made in only 1.

The proteins of the serum were precipitated with zinc sulfate and sodium hydroxide as described previously (5) and the iodine included with the precipitated proteins was measured by the Riggs and Man permanganate acid ashing method (15).

With this method the range of concentrations in normal humans is 4.0 to 8.0 gamma per cent with a mean value of 5.6 ± 1.3 gamma per cent. No statistical difference was found between normal males and normal non-pregnant females (16). Determinations have been made in duplicate, but owing to the difficulties of the visual titration end-point a maximum difference of 1.0 gamma per cent may occur between the 2 samples. For this reason differences of 1.0 gamma per cent between the averages of 2 successive determinations of serum precipitable iodine on the same patient do not indicate a significant change.

RESULTS

In Figure 1 the concentrations of serum precipitable iodines of 29 normal pregnant women ranged between 6.2 and 11.2 gamma per cent. The majority of the iodine concentrations were high normal or were above the upper limit of the

normal range of non-pregnant women (8.0 gamma per cent). Elevated values were noted as early as 3 and 6 weeks after conception. The concentrations of serum precipitable iodines did not increase during the subsequent course of pregnancy (Figures 1 and 2). In 2 instances maternal venous and umbilical blood serum had identical amounts of serum precipitable iodine. The elevation of serum precipitable iodine during pregnancy soon subsided after delivery (Figure 2). The drop occurred in some instances as early as 1 week postpartum. In 3 patients whose serum iodine was measured 2 to 4 months after delivery there was a further decrease.

In 10 instances women who threatened to abort or aborted had serum precipitable iodines low for normal pregnancy. In 6 of these patients the iodine concentrations were low in the normal range, 4.3 to 5.8 gamma per cent. One patient on 3 grains of desiccated thyroid miscarried when the serum precipitable iodine was 7.3 gamma per cent and aborted a second time when she was not

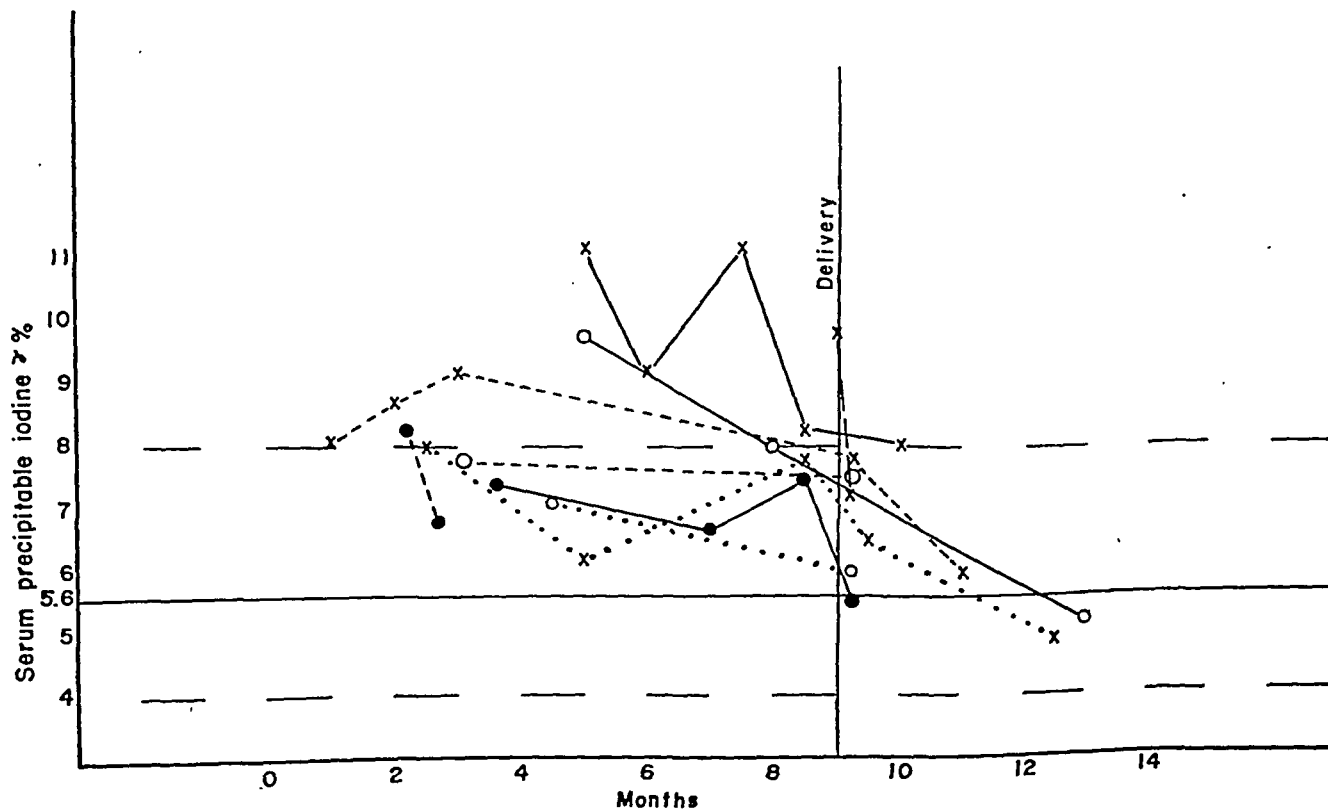


FIG. 2. SERUM PRECIPITABLE IODINES OF 9 WOMEN DURING AND AFTER PREGNANCY

Broken horizontal lines represent the upper and lower limits of normal range of serum precipitable iodine (4.0 to 8.0 gamma per cent). Solid horizontal line is 5.6 gamma per cent, the average normal value from statistical analysis.

Short broken line at $2\frac{1}{2}$ months connects concentrations before and 13 days after operative termination of cervical ectopic pregnancy.

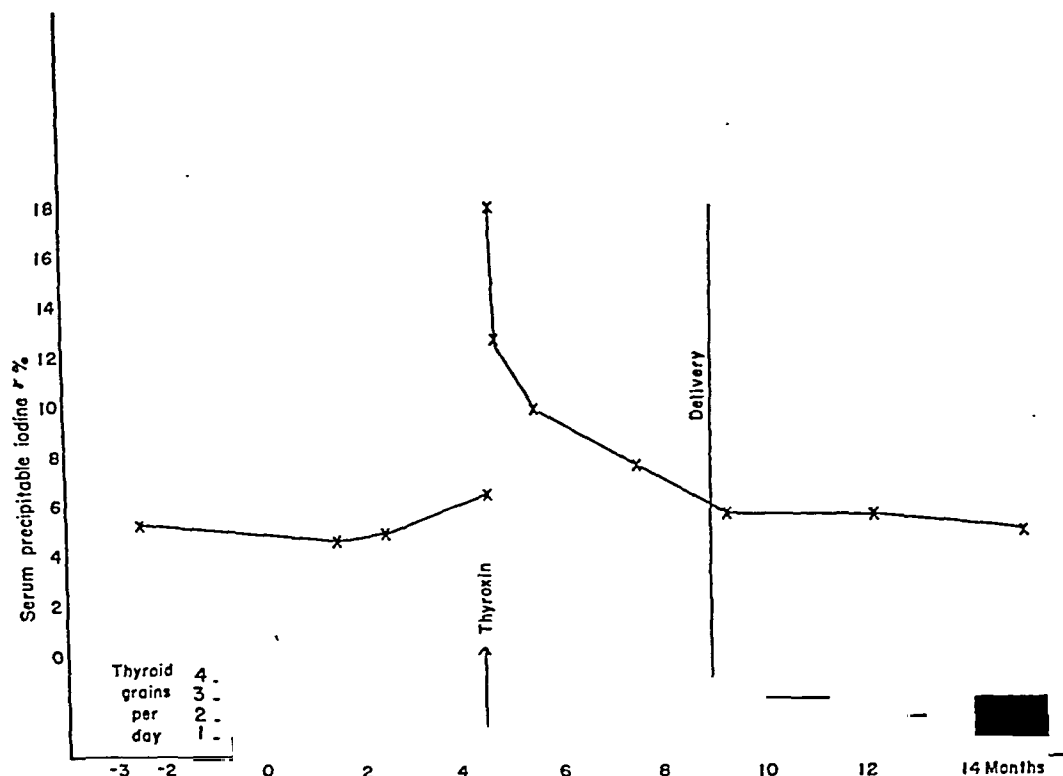


FIG. 3. SERUM PRECIPITABLE IODINES AND THYROID MEDICATION OF A HYPOTHYROID PATIENT DURING AND AFTER PREGNANCY

During the fourth month of pregnancy this patient developed labor pains and profuse vaginal bleeding. Shortly after hospitalization blood was taken for measurement of serum iodine. She was then given 2 mgm. of thyroxin intravenously. This was repeated the following day. The markedly elevated serum precipitable iodines occurred 1 and 6 days after the second intravenous injection of thyroxin.

taking thyroid and her iodine was as low as 2.8 gamma per cent. One patient (Figure 3) on $1\frac{1}{2}$ grains of desiccated thyroid daily threatened to abort when her iodine was 6.6 gamma per cent; another patient not on thyroid threatened to, but did not abort when her iodine was 7.3 gamma per cent. When such patients were given desiccated thyroid during pregnancy the serum iodine concentrations increased; but in some instances remained in the lower range of values of normal pregnancy (Figure 1).

The course of the serum precipitable iodine of a hypothyroid woman during pregnancy is shown in Figure 3. In the third month of pregnancy she took $1\frac{1}{2}$ grains of desiccated thyroid daily. In the fourth month she developed labor pains and began to hemorrhage so severely that in the opinion of the obstetrician an abortion was inevitable. The

serum precipitable iodine of blood taken immediately after hospitalization was 6.6 gamma per cent. She was given 2 mgm. of thyroxin intravenously on 2 successive days. Desiccated thyroid was increased to 3 and then 4 grains daily during the subsequent course of pregnancy; after the effect of intravenous thyroxin had subsided the serum precipitable iodines were 10.0 and 7.8 gamma per cent. She was delivered of a normal baby at term.

In 3 pregnant women with hyperthyroidism (Figure 4) higher concentrations of serum precipitable iodine occurred than in normal pregnant subjects. These subjects were treated with thio-urea and Lugol's solution. All delivered apparently normal babies at term. One patient with hyperthyroidism (not shown in Figure 4) was treated with Lugol's solution alone.

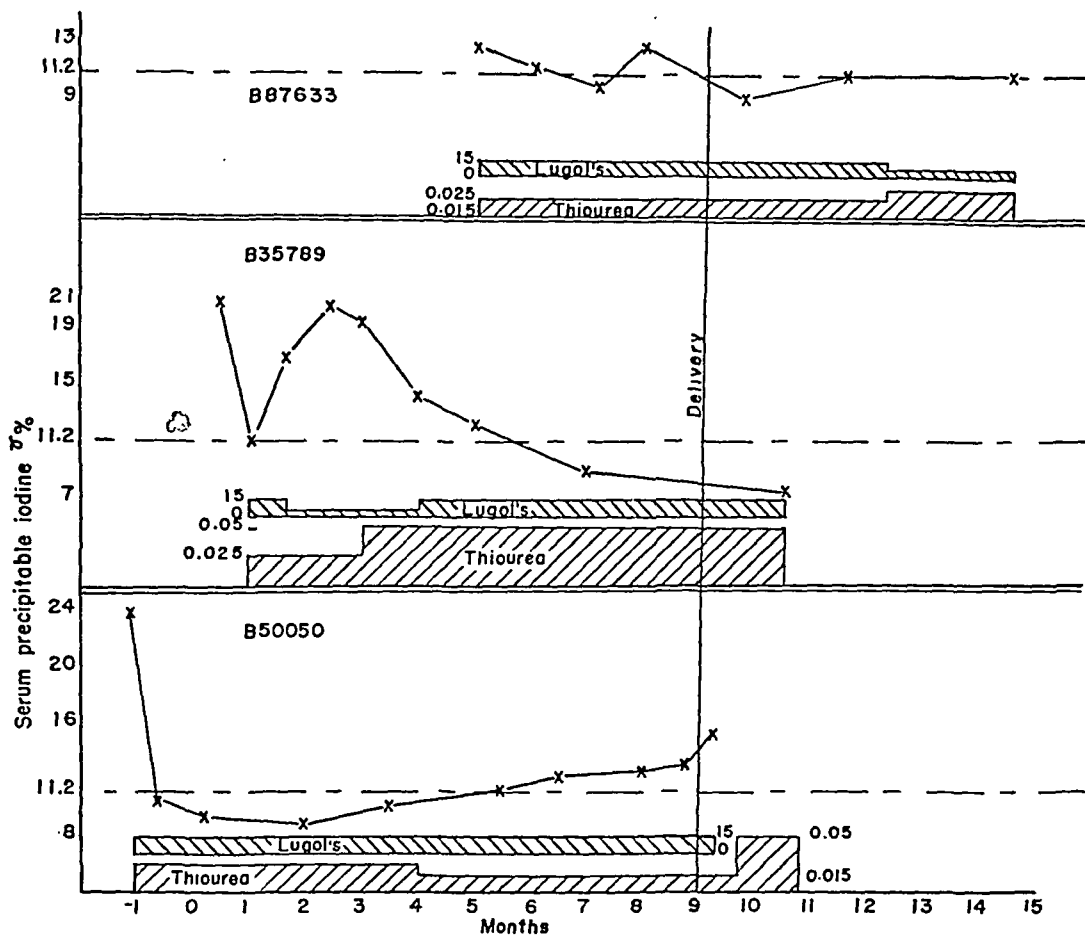


FIG. 4. SERUM PRECIPITABLE IODINES OF 3 HYPERTHYROID PATIENTS BEFORE, DURING, AND AFTER PREGNANCY ON THIOUREA AND LUGOL'S SOLUTION

Daily doses of thiourea are expressed in grams, Lugol's solution in drops. The broken horizontal lines represent the maximum value, 11.2 gamma per cent, of serum precipitable iodine observed in non-hyperthyroid pregnant women.

B50050 had a diffusely enlarged goiter with thrill and bruit, but no eye signs. After 12 days on 0.025 gram of thiourea and 15 drops of Lugol's solution daily her basal metabolic rate dropped from +68 to +15 per cent, her pulse decreased from 124 to 90 and her serum precipitable iodine fell from 24.2 to 10.2 gamma per cent. Three months after the beginning of medication it was recognized that she was pregnant. Two months later the thiourea dosage was decreased to 0.015 gram daily, but Lugol's solution was maintained at 15 drops daily. Her maximum serum precipitable iodine remained between 11.5 and 13.1 gamma per cent during pregnancy.

Hyperthyroidism with diffuse enlargement of the thyroid, fine tremor of tongue and fingers, but without eye signs, was recognized in B35789 when she was 2 weeks pregnant. After 2 weeks' medication with 0.025 gram of thiourea and 15 drops of Lugol's solution daily, clinical improvement was associated with a decrease in basal metabolic rate from +56 to +23 per cent, in pulse rate from 82 to 68, and in serum precipitable iodine from 21.1 to 11.2 gamma per cent. Subsequently the symptoms of hyperthyroidism increased and the serum precipitable iodine rose to 20.7 gamma per cent. Thiourea dosage was doubled to 0.05 gram per day. The patient was maintained on this dosage throughout pregnancy.

B87633 did not develop symptoms of hyperthyroidism until the fifth month of pregnancy. This patient had only mild symptoms of hyperthyroidism, the basal metabolic rate was +25 per cent. On a small amount of thiourea, 0.015 gram daily, and 15 drops of Lugol's solution her hyperthyroidism was controlled throughout pregnancy. Thiourea was increased and Lugol's solution was decreased 3½ months after delivery.

DISCUSSION

The elevation of serum precipitable iodine of pregnant women is not associated with clinical signs of hyperthyroidism or with demonstrable increases in oxidative processes. As mentioned before there were neither tachycardia, increased pulse pressure, eye signs, nor conspicuous changes in size or consistency of the thyroid gland. Previous reports from this laboratory (1, 5) have stressed that serum precipitable iodines above 8.0 gamma per cent indicate overactivity of the thyroid gland. In hyperthyroidism administration of Lugol's solution usually effects a drop in serum precipitable iodine (1, 5). In 2 women, one 2 months and one 6 months pregnant, 15 drops of Lugol's solution per day for 5 and 9 weeks, respectively, failed to cause any significant variation of serum precipitable iodine. Basal metabolic rates, one measure of oxidative processes, are not elevated before the latter part of pregnancy (11 to 14). The increase in serum precipitable iodine, however, was observed as early as 3 to 6 weeks after conception (Figure 1), and obviously is not correlated with the rise of basal metabolic rate. The increase of iodine during pregnancy is emphasized by the marked diminution following delivery (Figure 2). The elevation of serum precipitable iodine occurs whether the pregnancy be uterine or ectopic. The serum precipitable iodine of a woman with a 2½ month ectopic cervical pregnancy was 8.3 gamma per cent before, and 6.8 gamma per cent 13 days after operative termination of the pregnancy.

The thought of hyperthyroidism as a feature of normal pregnancy seems untenable. The elevations of serum precipitable iodine more likely constitute an increase in active circulating thyroid hormone which, however, does not affect tissue metabolism in the conventional manner. The normal gestatory changes in hormonal pattern might increase the pregnant organism's need for thyroid hormone and alter its response to it in such a manner that increased thyroid hormone concentrations do not cause a corresponding increase in cellular stimulation.

The patients in Figure 4 are examples of hyperthyroidism with a mild course during pregnancy. These 3 patients developed clinical symptoms of hyperthyroidism at different stages of

pregnancy. In comparison with the quantities of thiourea (0.04 and 0.28 grams daily) previously reported to control hyperthyroidism in non-pregnant subjects (17, 18), these women required small quantities of thiourea. A fourth patient with mild hyperthyroidism was controlled on Lugol's solution alone. Treatment of hyperthyroidism in pregnancy should not depress the serum precipitable iodines much below 8.0 gamma per cent. Miscarriages have been observed in some patients when serum iodine concentrations were low.

Another possible explanation for the increase in serum precipitable iodine during pregnancy would be that the technique of measuring precipitable iodine in serum from pregnant persons determines compounds chemically different from those in non-pregnant persons. This possibility cannot be excluded at this moment because analyses of the chemical nature of the compounds determined as serum precipitable iodine are not available. For instance organic iodine-containing substances like Priodax, Lipiodol and Diodrast are precipitated with proteins and cannot yet be distinguished from the endogenous iodine compounds of thyroid hormone. At present it would seem that the determinations reported here as serum precipitable iodine do not include fictitious iodine compounds. The increased values of serum precipitable iodine during normal pregnancy seem physiologic and may be as indispensable for the maintenance of pregnancy as changes in pituitary function and hormone concentration. Failure of hormonal adjustment typical of pregnancy will cause premature terminations (19). The intramuscular administration of 100,000 units of estradiol in oil within 1 week to 1 pregnant woman increased the serum precipitable iodine by 1.2 gamma per cent only. Another patient who threatened to abort in the third month of pregnancy was given 70 mgm. of stilbestrol intramuscularly. Two days later the serum precipitable iodine was 1.2 gamma per cent above the concentration (7.3 gamma per cent) before stilbestrol. In view of the magnitude of the experimental error these increases are equivocal. In some preliminary observations on normal women the serum precipitable iodine decreases from pre- to post-menstrual dates. The differences, however, are too small to prove serum iodine changes during menstrual cycles. Investigation of

such problems requires a more refined technique than is now available. David and Zener recently reported on "Influence of Iodine Therapy on Blood Iodine and Basal Metabolic Rate in Pregnancy" (20). Their data cannot be compared with ours because the range of normal values in their determinations is at variance with those of Talbot (8), Curtis (21), Taurog (22), and ours (16).

In women with serum precipitable iodine concentrations low for pregnancy it seemed logical to give desiccated thyroid by mouth or thyroxin intravenously. In some instances such therapy seemed to prevent abortions judged inevitable according to obstetrical criteria. It is not implied, however, that thyroid therapy is the treatment of choice for threatened abortion. The small number of observations in this series obviates statistical significance. While more data need to be collected trials with thyroid therapy are indicated if abnormally low serum precipitable iodine concentrations are established and other causes for abortion can be excluded. The effect of thyroid medication did not correspond to the one seen in the non-pregnant entity of hypothyroidism. The pregnant women were given doses of desiccated thyroid as large as 4 to 5 grains per day without any evidence of reduced tolerance for desiccated thyroid which is so typical for the hypothyroid patient (23). Such tolerance is similar to that of euthyroid persons (24). This suggests that certain tissues in the pregnant person require thyroid hormone in amounts larger than normal for some as yet unknown function, but unassociated with the overall calorogenic effect. For example, 3 patients (55164, B12368, and B29810), not on thyroid and without clinical signs of hypothyroidism, conceived normally, but threatened to abort between 2½ and 4 months after conception when their iodines were between 5.1 and 7.3 gamma per cent. Intravenous thyroxin or desiccated thyroid, 1 to 4 grains daily, was administered. They carried through pregnancy to delivery at term. Another example of thyroid medication during pregnancy concerns the 1 definite hypothyroid patient in the series. She was given more desiccated thyroid during pregnancy than either before conception or after delivery (Figure 3). During her first pregnancy in 1942 she took desiccated thyroid. Soon after delivery it was discontinued and the patient observed changes indicating hy-

pothyroidism. Without thyroid she subsequently became pregnant three times, but miscarried each time. During the pregnancy shown in Figure 3, although she was on 1½ grains of desiccated thyroid daily she had vaginal bleeding and labor pains at 4½ months. After intravenous thyroxin the bleeding and pain stopped. On 3 and then 4 grains of thyroid daily she carried through pregnancy and delivered a normal baby at term. After delivery 3 grains of thyroid daily seemed sufficient, clinically, to control her hypothyroidism.

That patients conceive readily without thyroid medication is suggested by the histories of the 4 previous patients. Observations on them support the theory that thyroid therapy for infertility should be restricted to instances of hypothyroidism. This is equally true for such therapy in menstrual disorders. Data on 1 patient need confirmation. These data may represent the behavior of the serum precipitable iodine in these patients who conceive readily, threaten to abort and then, after thyroid administration, carry through pregnancy. This patient was one who not only failed to develop the increase that usually occurs with pregnancy, but showed a fall in serum iodine. Before conception the serum precipitable iodine was 4.1 gamma per cent. She became pregnant and miscarried in the third week, at which time her serum precipitable iodine was only 2.8 gamma per cent. Three weeks later, still without thyroid medication, the serum precipitable iodine had risen to 4.4 gamma per cent. In this instance pre- and post-pregnant iodine concentrations were normal. Thyroid medication did not seem to be indicated either clinically or biochemically.

CONCLUSIONS

Serum precipitable iodines of 43 pregnant women have been measured during pregnancy and in 11 instances after delivery.

In 29 normal pregnancies, serum precipitable iodines ranged between 6.2 and 11.2 gamma per cent, the normal non-pregnant range being 4.0 to 8.0 gamma per cent. This elevation of serum precipitable iodine was noted as early as 3 weeks after conception, did not increase as pregnancy advanced and decreased rapidly to the normal range after delivery. Elevated serum precipitable iodines are physiologic for normal pregnancies; they do not indicate hyperthyroidism.

In some patients who threatened to abort, or actually aborted, the serum precipitable iodines were between 2.8 and 5.8 gamma per cent. In such instances the administration of desiccated thyroid should be considered. Thyroid medication for infertility appears to be indicated only in hypothyroid patients.

Hyperthyroidism of 4 pregnant women was controlled easily and pregnancy maintained with Lugol's solution alone, or in conjunction with small amounts of thiourea.

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STUDIES OF HEMOPHILIA. II. THE ASSAY OF THE ANTIHEMOPHILIC CLOT-PROMOTING PRINCIPLE IN NORMAL HUMAN PLASMA WITH SOME OBSERVATIONS ON THE RELATIVE POTENCY OF CERTAIN PLASMA FRACTIONS¹

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Ever since Sahli (1) demonstrated that the coagulation of hemophilic blood is accelerated by the addition of normal blood² many workers (2 to 9) have focused attention on its clot-promoting property. Since cell-free plasma seemed as effective as platelet-rich plasma this action could not be directly attributed to any of the cellular components of blood (2). Hemophilic plasma was found to be inert.

The antihemophilic factors in normal plasma seem to be associated with the globulins and fibrinogen (3 to 7), and appear preponderantly in Fraction I obtained by Cohn and his collaborators (7, 10 to 12).

The present communication deals with the quantitative relationship between the amount of normal plasma added to hemophilic blood and the coagulation time of such mixtures. We believe that the observations provide a basis for estimating antihemophilic activity of plasma, and we present them in the hope that they will be extended by others in studies of the coagulation defect of hemophilic blood. Also included are some observations on the potency of certain plasma fractions, which demonstrate the applicability of the relationship to work aimed at the separation of the clot-promoting principle from plasma.

METHOD

Five hemophiliacs were studied, 4 adults and 1 child. The clinical manifestations and laboratory tests conformed entirely with a diagnosis of hemophilia.

Coagulation times were determined by a modification of the Lee and White method (13). No determination was considered valid unless the hemophiliac's vein was entered directly with the first puncture and blood flowed readily into the syringe.

¹ Presented in abstract form before the American Society for Clinical Investigation at its annual meetings in 1946 and 1947.

² Except where otherwise indicated, the term "normal" will refer to "non-hemophilic."

Normal plasma was obtained by adding 9 parts (by volume) of venous blood, drawn under stasis, to 1 part (by volume) of 2.5 per cent sodium citrate solution, centrifuging the mixture promptly at 2000 r.p.m. for 10 minutes, and removing the supernatant citrated plasma. Serial dilutions were prepared immediately before testing by diluting with a volume of physiological saline sufficient to contain the desired amount of plasma to be tested in 0.1 cc. of the diluted mixture. This was pipetted into the clotting-time tubes previously rinsed with physiological saline, 2 cc. of freshly drawn hemophilic blood were added, and the coagulation times were recorded.

OBSERVATIONS

Clotting time of hemophilic blood mixed in vitro with varying amounts of normal plasma

The coagulation of hemophilic blood added to minute amounts of normal plasma was accelerated to a degree which depended on the relative amount of normal plasma in the mixture (Figure 1). Although this reaction was uniform for all the patients studied, each hemophiliac exhibited a pattern peculiar to himself.

When the logarithm of the clotting time was plotted against the logarithm of the amount of normal plasma added to 2 cc. of hemophilic blood a linear relationship was obtained (Figure 2) which, in 3 hemophiliacs who were thus studied, was relatively constant over a period of from 10 to 23 months of observation, despite the fact that these subjects had been receiving plasma infusions 3 to 4 times weekly as prophylactic therapy (14).

Plasma from different individuals (Table I) or from the same individual at different times vary somewhat in antihemophilic activity. In 46 non-hemophilic plasmas³ tested on the blood of the same hemophiliac over a period of 15 months the

³ Comprising fresh or freshly processed frozen plasmas from normal male and female subjects and from patients with anemia, polycythemia, myocardial infarction, cancer, infections.

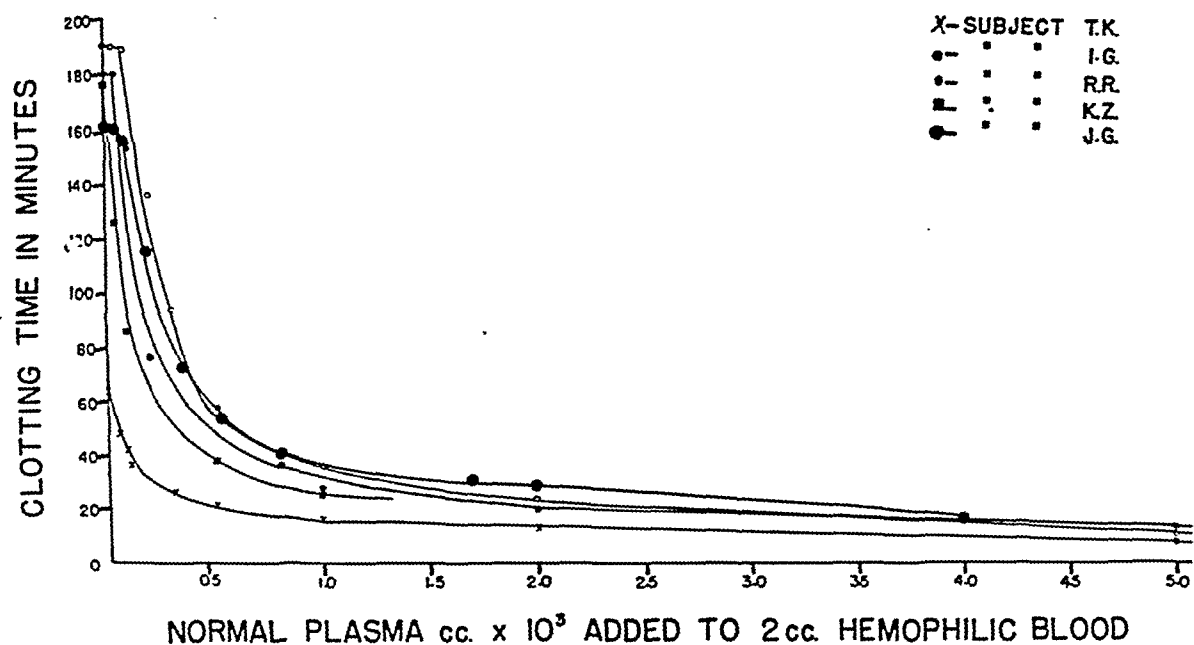


FIG. 1. RELATIONSHIP BETWEEN THE AMOUNT OF NORMAL CITRATED PLASMA ADDED *IN VITRO* TO HEMOPHILIC BLOOD AND THE COAGULATION TIME OF THE MIXTURE

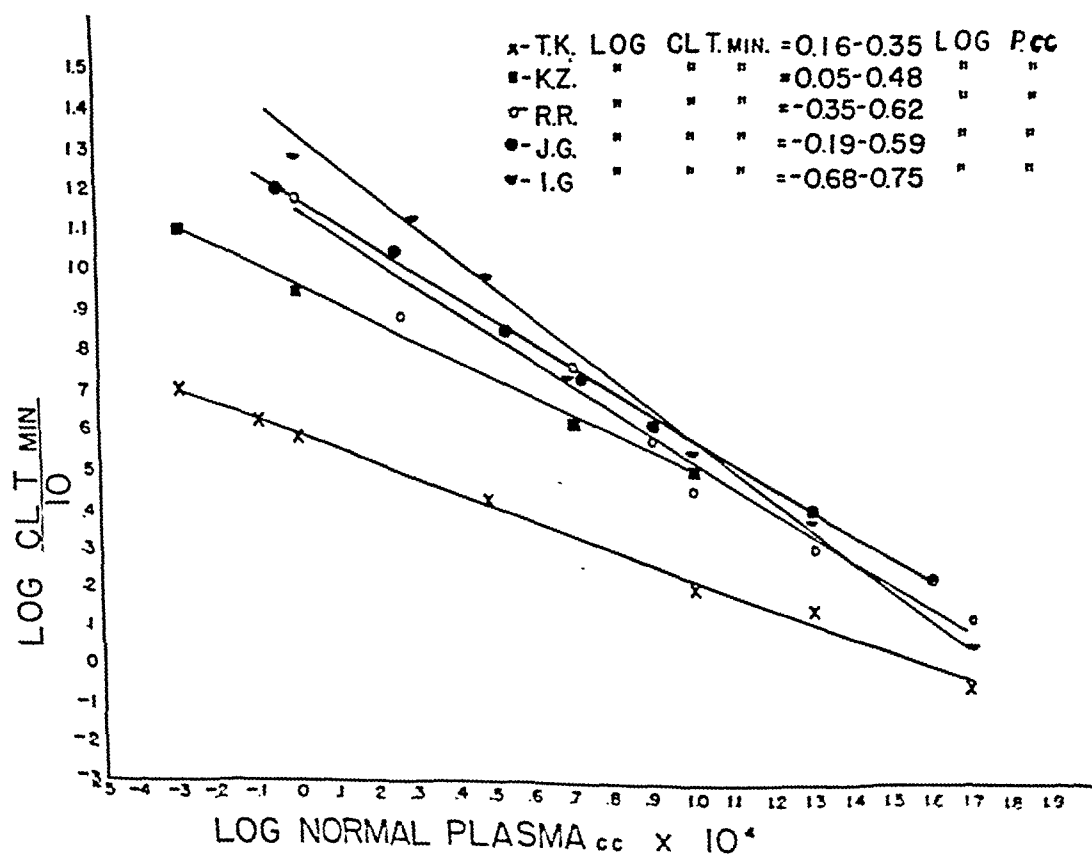


FIG. 2. LOGARITHMIC PLOT OF RELATIONSHIP PRESENTED IN FIGURE 1

TABLE I

Effect of normal plasma from different individuals on coagulation time of hemophilic blood

Subject	Clotting time (min.) after addition of plasma to 2 cc. hemophilic blood		
	Amount of plasma added		
	0.001 cc.	0.0005 cc.	
Experiment 1			
Hemophilic T. K. Control Cl. T., 65 min.			
Mr. Sp.	0→	16.5	23
Mr. Sh.	0→	16.5	21
Mrs. R.	0+	17.5	23
Experiment 2			
Hemophilic R. R. Control Cl. T., 64 min.			
G. L.	0+	30	36
B. A.	0→	28	36
Experiment 3			
Hemophilic T. K. Control Cl. T., 34 min.			
G. L.	0+	14.5	19.5
B. A.	0→	17.5	21.0
Experiment 4			
Hemophilic T. K. Control Cl. T., 33 min.			
Miss I.	0+	17.0	24.5
Dr. Hu.	0→	16.5	24.0
Dr. Ho.	0→	19.0	26.0
Miss W.	0+	17.5	24.5
Experiment 5			
Hemophilic I. G. Control Cl. T., 42 min.			
Mr. Sh.	0→	19	27
Mr. G.	0→	21	29

potency was fairly uniform with a standard deviation of 0.3 unit in terms of a mean activity of 1.0 unit.

Correlation between the original coagulation time, clotting time induced by addition of normal plasma, and the clinical severity of the hemophilia

There seemed to be some degree of correlation between the clinical severity of the hemophilia and the slope of the relationship shown in Figure 2. Thus T. K. (original clotting time 70 min.) with a slope of -0.35 , was the mildest hemophiliac (had suffered the least crippling hemorrhages) of our series, whereas I. G. (original clotting time 190 minutes) with a slope of -0.75 , was the worst. R. R. and J. G. (original clotting times 180 and 170 to 200 minutes, respectively) with slopes of -0.62 and -0.59 were moderately severe hemophiliacs clinically.

TABLE II

Assay of antihemophilic potency of Fraction I obtained from human plasma*

Test subject	Orig. cl. t.	Lot No.	Amt. added to 2 cc. hem. bl.	Final cl. t.		Antihemophilic activity in per cent of that in an equivalent amount of normal plasma	Average antihemophilic activity
				min.	min.		per cent
T. K.	62	G20	0.002	29	15	17.5	15.8
			0.001	41	19.5	14.1	
		G35	0.002	38	15	8.5	7.6
			0.001	55	19.5	6.6	
R. R.	113	G20	0.002	48	20.4	21	19.5
			0.001	67	30.2	18	
		G35	0.002	51	20.4	17.5	13.2
			0.001	81	30.2	9.0	
I. G.	72	G20	0.002	38	21.8	25.0	
			0.001	50	28.2	22.0	23.5
		G35	0.002	48	21.8	13.0	
			0.001	63	28.2	7.0	10.0
R. R.	112	G15	0.001	54	30.2	31.2	35.3
			0.0005	65	43.6	39.4	

* See footnote 4.

† Solution containing 0.20 gram Fraction I in 67 cc. physiological saline. This volume was used since it represents the volume of citrated plasma from which the 0.2 gram of Fraction I was derived.

TABLE III

Antihemophilic potency of plasma protein fraction precipitated by CO₂ at pH 5.74

Experiment 1

Test subject R. R. Clotting time, 87 minutes

Fraction * tested	Cl. t.	Antihemophilic activity
	min.	per cent
I. Parent plasma	29	100
II. CO ₂ precipitate	29	100
III. CO ₂ supernatant	63	14
IV. Repeat precipitation of II with CO ₂	70	8
V. Supernatant of IV	61	16

Experiment 2

Test subject R. R. Clotting time, 87 minutes

I. Parent plasma	25	100
†II. CO ₂ precipitate	23	105
III. CO ₂ supernatant	60	13

* To 2 cc. of hemophilic blood was added an amount of material derived from 0.001 cc. of plasma in a volume of 0.1 cc.

† An amount of this material (about 500 mgm.) derived from 90 cc. of plasma was given intravenously to the same patient. Three minutes after injection the clotting time was reduced from 87 to 13 minutes; 1½ hours later the clotting time was 22 minutes, and 20 hours later it was 44 minutes. About ½ hour after injection the patient felt hot all over. This, the only untoward symptom, disappeared in 5 minutes.

Determination of the anti-hemophilic potency of certain plasma fractions

The coagulation-accelerating property of plasma fractions can be assayed by comparing their anti-hemophilic activity with the parent plasma from which they were derived or with the mean activity of many plasmas. The results of illustrative examples of experiments in which this was done are given in Tables II and III. Samples of plasma Fraction I⁴ were dissolved in a volume of physiological saline equal to that of the plasma from which the fraction was derived. A dilution of this was made with sufficient saline so that 0.1 cc. of the final dilution would contain an amount of anti-hemophilic material comparable in activity to no more than 0.001 cc. of plasma. With Fraction I it was impossible to compare the activity with that of the parent plasma since the latter was not available. Accordingly, its potency was calculated in terms of per cent of the mean potency of normal plasma.

Another plasma protein fraction (Table III) was prepared by CO₂ saturation of citrated plasma diluted 1 to 10 (by volume) with distilled water. Fractionation was carried out at 0 to 5° C., and the pH after saturation was 5.74. A few droplets of caprylic alcohol were added to prevent foaming. The precipitate, separated from the supernatant by centrifugation, was redissolved in a volume of 1.5 per cent sodium chloride solution one-tenth the original volume of plasma. To an aliquot sample was added 9 volumes of physiological saline. Sufficient solid sodium chloride was added to the supernatant to make it isotonic. A sample of the parent plasma, kept beside the material being fractionated, the plasma precipitate, and the supernatant were tested simultaneously against hemophilic blood.

The results indicate that several samples of Fraction I contained only 8 to 35 per cent of the anti-hemophilic activity of average plasma. On the other hand, the anti-hemophilic principle in normal plasma was quantitatively precipitated by CO₂ under the above conditions. The precipitate was also active *in vivo*. Re-precipitation with CO₂ resulted in large losses of anti-hemophilic potency.

⁴ Obtained through the courtesy of Dr. John T. Edsall, Dept. of Physical Chemistry, Harvard Medical School, from the Mass. State Division of Biologic Laboratories.

DISCUSSION

The cardinal disturbance in hemophilia is the retarded coagulation of hemophilic blood. Both the explanation of this phenomenon and the mechanism whereby normal plasma rectifies it are still unknown. Accordingly, the quantitative relationships observed by us between the addition of normal plasma to hemophilic blood and the clotting time of such mixtures must remain empiric.

Extremely minute amounts of plasma have a profound effect in accelerating the coagulation of hemophilic blood. As the amount of plasma is increased and the coagulation time approaches normal the increment in further reduction of the clotting time becomes increasingly less until it is no longer measurable. From inspection of the curves (Figures 1 and 2) it is obvious that differences in anti-hemophilic activity of between 0.0005 and 0.001 cc. of normal plasma are reflected by greater differences in clotting time than between 0.001 and 0.002 cc. when these volumes of plasma are added to 2 cc. of hemophilic blood. Clot acceleration induced by 0.002 cc. is almost maximal. The effects of 0.003, 0.004, and 0.005 cc. or more are practically indistinguishable from that of 0.002 cc. since the differences between the acceleration of coagulation induced by each are almost within the limits of accuracy of the method.

A clear realization of this is essential in studies on the anti-hemophilic clot-promoting moiety of human plasma or of other biologic sources. Unfortunately most of the reported investigations are based upon methods in which the clot-promoting effects of no less than 0.01 cc. of plasma, or its equivalent, are observed. Under such conditions losses in anti-hemophilic activity incurred in plasma fractionation procedures may have escaped detection, and erroneous conclusions may have been drawn.

Clearly the relationships indicated in Figures 1 and 2 are empirical observations which hold only between 2 limits. The one is imposed by the clotting time of hemophilic blood to which no normal plasma has been added and the sensitivity of the method in detecting acceleration in coagulation consequent to the addition of infinitesimally small amounts of normal plasma. Increasing the relative amount of normal plasma in the plasma-hemophilic blood mixture results in progressively

greater overall reduction in coagulation time until the relatively normal range is reached asymptotically. This is the other limit in the relationship.

The configuration of the curves throws little light on the nature of the antihemophilic principle. Since the curves relating the clotting time of hemophilic blood to the amount of plasma added to it (Figure 1) resemble in shape the curves relating the activities of thrombin, prothrombin, and thromboplastin to their concentrations (15), it would appear that normal plasma restores to hemophilic blood normal coagulation processes involving some or all of these elements. That the effects are not referable to the addition of thrombin *per se* is obvious since plasma contains no thrombin. Prothrombin also cannot be implicated since prothrombin-free plasma retains antihemophilic activity (16). There is, furthermore, no evidence of a quantitative or qualitative prothrombin defect in hemophilia (8). Whether one prefers to label the antihemophilic moiety "plasma thromboplastin," as did Howell (17), or "globulin substance" as does Taylor *et al.* (3, 12) is academic since, until more is known regarding its physiological and biochemical properties, we can refer to it only through its effects on hemophilic blood.

The shape of the curves tells us little regarding the dynamics of the reaction between normal plasma and hemophilic blood. The linear nature of the logarithmic relationship does, however, indicate the reaction to be of an order higher than unimolecular. This curve, by being easily expressed mathematically, lends itself readily to calculation of the clotting time expected of any given normal plasma-hemophilic blood mixture. Contrariwise, the amount of normal plasma, or its antihemophilic equivalent, in such a mixture may be computed from its coagulation time. The value of this relationship is apparent in studies aimed at separation and purification of the antihemophilic principle of normal plasma, and elucidation of its biochemical and physiological properties.

Besides being of considerable clinical interest and significance the constancy of this relationship between clotting time and the amount of normal plasma is also useful. Since the effect on the coagulation of hemophilic blood is the only property by which the antihemophilic principle can now be measured, a well standardized hemophiliac can provide a readily available source of test material

for quantitative studies. Furthermore, the pattern of his reaction remains unaffected by prophylactic treatment with plasma.

It should not be inferred from this that the coagulation time of any particular hemophiliac remains constant. That the clotting time may fluctuate considerably from time to time is well known. Should this occur the patient can be readily re-standardized. Furthermore, as can be seen from our data, an extremely minute alteration in the amount of antihemophilic principle present in a fixed volume of hemophilic blood will produce a profound change in coagulation time in the steep part of the curve where coagulation times are greatest. Thus, for example, subject T. K. (Figure 1) has a clotting time which has spontaneously varied from 65 to 90 minutes. Although this discrepancy appears large it represents the activity of an extremely minute amount of antihemophilic principle in this range of the curve, which, if disregarded, introduces a negligible error.

It should be pointed out that acceleration of the coagulation of hemophilic blood is not an effect produced exclusively by the antihemophilic principle of plasma. Other plasma or tissue derivatives are also effective in this regard. Small amounts of tissue thromboplastin, or of thrombin, added to hemophilic blood will speed its coagulation. Extremely minute contamination of hemophilic blood with tissue juice, as can occur in unsuccessful venipuncture, will give erroneously low, if not normal, coagulation times (2), presumably due to tissue thromboplastin. Other agents capable of promoting the coagulation of hemophilic blood are certain proteolytic enzymes (18, 19). Accordingly, it is necessary to exclude, by suitable tests, the possibility that the clot-promoting effect of any biologic preparation is referable to any of the above mentioned substances.

Attention should be called to certain other advisable precautions and refinements in technique which would undoubtedly enhance the accuracy of the method. Our failure to recognize them early in the course of this study probably accounts in part for the substantial size of the standard deviation of normal plasma:

1. *Accurate delivery of measured amount of hemophilic blood.* Undoubtedly, a certain degree of inaccuracy arises from difficulty in placing ex-

actly 2.0 cc. of hemophilic blood in the clotting-time tube, particularly when large numbers of determinations are being made simultaneously and blood must be delivered rapidly from a 50-cc. syringe. We have found that, even when care is exercised, up to 15 per cent too much or too little blood may be added, which may result in errors of this magnitude. This can be obviated by using graduated clotting-time tubes and by filling with blood exactly to the 2.1 cc. mark (0.1 cc. of test material or saline having first been added) or by estimating the 2 cc. of blood and subsequently recording the exact amount by reading it off from the calibrated tube. Due correction can then be applied.

2. *Red cell hemolysis.* From observations we have made, to be reported in a subsequent communication, we have found that hemolysis of red cells, both normal and hemophilic, liberates a substance which will hasten the coagulation of hemophilic blood. The material is probably related to cephalin and exhibits thromboplastic activity. Accordingly, hemolysis in the drawing of hemophilic or normal blood should be avoided as much as possible.

3. *Errors arising from admixture of tissue juice to blood samples.* The invalidation of tests in which hemophilic blood is drawn by venipunctures which are not immediately successful has been mentioned. Similarly, in the drawing of normal blood for standardization of a particular hemophiliac, care should be exercised that the donor's vein be entered by "primary intention" in order to exclude tissue juice in test samples.

4. *Errors arising from delay in completing experiments.* The antihemophilic principle of normal plasma deteriorates quite rapidly in liquid plasma (20). The lability is especially marked in whole blood. Accordingly, substantial errors may arise from delay in separating the plasma from the cellular blood components and/or delay in running the experiments after the plasma has been obtained.

From our assay of the antihemophilic potency of some specimens of Fraction I of plasma, it appears that most of the antihemophilic activity had been lost in their preparation. Three different samples of this fraction exhibited only about 20, 10, and 35 per cent of the potency of normal plasma. The loss may only be apparent, and it may in part be

attributable to low potencies of the parent plasmas resulting from deterioration consequent to unavoidable delays in processing. Some loss incurred in fractionation is, however, indicated by the appearance of clot-promoting activity in Fractions II and III (12).

There is remarkably little information in the literature concerning the antihemophilic potency of Fraction I and other plasma protein fractions, especially as compared with the original plasmas from which they were derived. From the most recent publication in this regard, by Taylor *et al.* (12), it would appear that Fraction I contains most of the antihemophilic activity to be found in plasma.

It is possible that the samples of Fraction I tested by these investigators was more potent than those assayed by us. Since, however, they tested no less than that amount of Fraction I⁵ derived from approximately 0.01 cc. of plasma it is possible that the discrepancy in reported potency of this plasma protein fraction may be referable to their use of a less sensitive range in the measurement of antihemophilic activity.

That the antihemophilic component of normal plasma is precipitated practically quantitatively by CO₂ saturation of diluted plasma, reported by others (3, 21) and confirmed by us, is of practical importance. The full activity of 100 cc. of plasma is contained in about 500 mgm. of this protein fraction. Five hundred mgm. of Fraction I, on the other hand, appear to contain the antihemophilic activity of only 13 to 60 cc. of plasma. The CO₂ precipitated fraction would accordingly seem to be a better starting point for further purification of the antihemophilic principle than Fraction I.

The importance of comparing clot-promoting activity of plasma fractions with their parent plasmas, or, these being unavailable, with the mean activity of normal plasma has been referred to above. We feel that it is desirable, in plasma fractionation work, to consider normal plasma arbitrarily as a standard of reference. We propose that 0.001 cc. of plasma be designated as containing 1 unit of antihemophilic activity. This amount of plasma is selected arbitrarily because it also represents an amount of antihemophilic activity

⁵0.1 cc. of 1:100 dilution of a 4 per cent solution of Fraction I. Four grams of Fraction I are derived from 1000 cc. of plasma (10).

which should not be exceeded in *in vitro* assay on hemophilic blood. On this basis relative degrees of purity and total yield of various plasma fractions can be readily expressed (Table IV).

TABLE IV
Comparative antihemophilic activity of 2 plasma protein fractions

Plasma fraction	Antihemophilic activity units*	Amount of antihemophilic material obtainable from 1 liter of citrated plasma	
		units	grams
Plasma	17	1,000,000	60
Fraction I		100,000 to 350,000	4
G-35	30		
G-20	60		
G-15	120		
CO ₂ precipitate	200	1,000,000	5

* One unit is defined as that amount of antihemophilic principle which accelerates the coagulation of 2 cc. of hemophilic blood to the same extent as 1 cu. mm. of average fresh citrated human plasma.

As little as 0.5 μ g. of the CO₂ precipitated fraction added to 2 cc. of hemophilic blood will accelerate its coagulation appreciably. The clot-promoting principle, which in its crude form is now active in a dilution of about 1 part in 2.2 million, will probably be found to exhibit much higher activity when it is purified further since the CO₂ precipitated fraction undoubtedly contains some fibrinogen and prothrombin (3, 21, 22).

SUMMARY AND CONCLUSIONS

1. The quantitative relationship between the coagulation time of shed hemophilic blood and the amount of normal plasma added to it is described. It provides the basis for a method of assaying the antihemophilic principle of plasma.

2. This relationship was peculiar to, and constant in, each of 4 hemophiliacs studied over a prolonged interval. It was unaffected by plasma therapy.

3. The antihemophilic activity of normal plasma is fairly uniform.

4. The clot-promoting principle of normal plasma is precipitated quantitatively from diluted plasma by saturation with CO₂. The precipitate is active in a dilution of 1 part in 2.2 million.

5. Three samples of plasma protein Fraction I were found to contain about 10, 20, and 30 per cent, respectively, of the antihemophilic potency of whole plasma.

6. It is proposed that whole normal plasma be considered a reference standard in studies aimed at concentration and purification of its antihemophilic principle. One antihemophilic unit is defined.

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LIVER INVOLVEMENT IN INFECTIOUS MONONUCLEOSIS¹

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The occurrence of hepatitis in the absence of jaundice in infectious mononucleosis has recently been emphasized. In 1946 Cohn and Lidman (1) published results of serial liver function studies carried out on 15 successive hospitalized patients with proved infectious mononucleosis in which there was neither clinical nor laboratory evidence of jaundice. All cases showed evidence of hepatic dysfunction as shown by abnormalities in more than 1 test. Carter and MacLagan (2) have studied 19 cases of infectious mononucleosis only one of which was jaundiced, and found elevated values for the thymol turbidity test in the serum of 58 per cent and a positive serum colloidal gold reaction in 95 per cent.

Since no serial study of the cephalin-cholesterol flocculation test in the serum of non-jaundiced infectious mononucleosis cases had been published, it was thought of interest to compare the results of this test in such serum with those of other measures of liver function.

MATERIALS AND METHODS

Serial determination of liver function tests were made on 19 consecutive cases of infectious mononucleosis without jaundice, 13 of which were hospitalized and 6 of which were ambulatory.² Determination of sheep RBC agglutination was based on the method of Stuart (3) and agglutination in a final serum dilution of 1:160 or higher was considered significant. By this criteria, all except 2 cases had elevated heterophile antibody titers: one of these, at 1:80, showed guinea pig kidney and beef RBC absorption characteristics of the infectious mononucleosis antibody, and the other had a blood picture and a clinical course typical of infectious mononucleosis.

Cephalin-cholesterol flocculation tests were done by the technique of Hanger (4) and a reaction of 2+ or more in 24 hours was considered abnormal. The thymol

turbidity test was performed by MacLagan's method (5) and up to 4.0 units taken as normal. Serum bilirubin including the 1-minute determination followed the method of Malloy and Evelyn (6): normal values were taken as those up to 0.31 and 1.20 mgm. per cent for the 1-minute and total values, respectively. Four Bodansky units were accepted as the upper limit of normal for the alkaline serum phosphatase determination, utilizing his technique (7). In the few instances in which brom-sulfalein excretion was measured, 5 mgm. per kilogram of body weight of the dye was injected and a value of 10 per cent or less was considered normal in the sample of serum drawn at the end of 30 minutes. Electrophoretic analysis of serum proteins³ was performed on 2 sera using the Tiselius apparatus, a barbiturate buffer of 0.1 ionic strength, and at a pH of 8.6.

RESULTS

The severity of the disease, the mononuclear percentage, the heterophile antibody titer, and the results of liver function tests, each shown according to the approximate day of the disease on which they were performed, are outlined in Table I, and in Figure 1 the percentage of cases whose sera showed abnormalities in liver function tests is compared with similar studies performed serially on the serum of patients with upper respiratory infections in which the diagnosis of infectious mononucleosis had been carefully excluded.

The cephalin-cholesterol flocculation test was abnormal in 95 per cent of the serum from patients with infectious mononucleosis and in 2 instances was altered before the heterophile antibody titer had reached a significant level. The 2 cases whose serum showed a normal flocculation reaction both had a mild illness during which they remained completely ambulatory. In contrast, none of the serum from 22 cases of upper respiratory infections, all of whom were ill enough to be hospitalized, demonstrated an abnormal cephalin flocculation test.

Elevated values for the thymol turbidity test

¹ Representing work done for the Virus and Rickettsial Disease Commission, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

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³ Electrophoretic analysis of serum proteins was kindly carried out by Dr. John Milne, Department of Internal Medicine, Yale University School of Medicine.

were found in 68 per cent of the serum from patients with infectious mononucleosis and ranged from 4 to 10 units. Abnormalities in this test usually appeared later and were more transient than the changes in the cephalin flocculation reaction. One sample of serum from a patient with an upper respiratory infection showed a slightly increased value for the thymol turbidity test.

Forty-three per cent of the serum of infectious mononucleosis patients showed values for the alkaline phosphatase test from 4 to 11.5 Bodansky units. No serum from cases of infectious mononucleosis nor from those with respiratory conditions demonstrated significant increases in the

total serum bilirubin determination, but slight increases in the 1-minute value above the arbitrary normal of 0.31 mgm. per cent were seen in both. Serial studies of bromsulfalein excretion were carried out in only 1 case of infectious mononucleosis and demonstrated a peak retention of 44 mgm. per cent in the 30-minute sample.

In Table II the results of analysis of serum proteins from a case of infectious mononucleosis without jaundice are compared with similar analysis of a serum from a jaundiced case, both of which were obtained approximately 1½ months after onset of illness. Marked increases in the percentages of beta and gamma globulin were observed in both.

TABLE I
Results of liver function studies in infectious mononucleosis cases without jaundice

Case	Day of disease	Severity of disease	Mono-nuclears	Heterophile antibody	Thymol turb.	Cephalin flocculation		Serum bilirubin		Alkaline phosphatase	BSP (30-min.)
						24	48	1 Min.	Total		
			<i>per cent</i>	<i>titer</i>	<i>units</i>			<i>mgm.</i>	<i>per cent</i>	<i>Bodansky units</i>	<i>per cent</i>
1	9	Severe		1:1280	2.5	0	1	0.07	0.48		
	11		23	1:1280	2.0	3	4	0.37	1.02	5.50	
	15		68	1:2560	2.0	3	3	0.51	0.94	5.35	
	29		53	1:640	4.5	3	3	0.41	0.88	4.32	
	32			1:320	6.0	3	3	0.61	1.63	2.87	
	45			1:160	4.5	3	3	0.34	0.61	2.08	
2	10	Severe	80	1:2560	5.5	3	4	0.03	0.75	2.65	
	14		72	1:1280	5.5	3	4	0.48	0.88	3.33	
	30			1:320	5.0	2	3				
3	2	Severe	53	1:40	2.0	3	4	0.10	1.20		
	11			1:2560	4.5	3	4	0.24	1.22	1.43	
	15			1:1280	3.0	3	4	0.20	0.75	2.73	8.4
	21			1:1280	7.5	4	4	0.20	1.09	2.78	
4	5	Severe	49	1:640	4.5	3	4	0.07	0.27		
	9			1:160	3.0	3	4	0.27	0.61	5.95	2.0
5	30	Severe	61	1:1280		3	4				
	38		68	1:1280	2.0	3	4	0.07	0.27		0
	44			1:1280	4.5	1	2				
	48			1:1280	5.5	±	1	0.07	0.41		
6	7	Severe	54	1:80	2.0	2	3	0.34	0.95	4.23	18
	10		70	1:640	2.5	4	4	0.10	0.28		
	16			1:1280	6.0	3	3	0.44	1.16	11.50	44
	41			1:640	1.0	4	4	0.07	1.16	3.75	13
7	8	Severe		1:640	6.0	2	4				
	15			1:640	10.0	4	4	0.10	0.28	2.18	1.5
8	5	Moderate	65	1:160	2.5	3	4	0.14	0.34		
	8			1:640	5.5	4	4	0.14	0.38		
	17			1:1280	6.0			0.37	1.07	3.10	
9	12	Moderate	61	1:640	3.0	2	3	0.58	0.93	9.77	
	53			1:80	2.0	0	0	0.10	0.34		
10	8	Moderate	89	1:640	5.0	4	4	0.24	0.61	7.75	
	11		77	1:640	2.5	3	4	0.07	0.41	2.64	

TABLE I—Continued

Case	Day of disease	Severity of disease	Mono-nuclears	Heterophile antibody	Thymol turb.	Cephalin flocculation		Serum bilirubin		Alkaline phosphatase	BSP (30-min.)
						24	48	1 Min.	Total		
			<i>per cent</i>	<i>titer</i>	<i>units</i>			<i>mgm.</i>	<i>per cent</i>	<i>Bodansky units</i>	<i>per cent</i>
11	17	Moderate	91	1:1280	7.0	4	4	0.07	0.42		
12	10	Moderate		1:1280	5.0	3	4	0.17	0.48	2.95	
13	7	Moderate	70	1:40	3.0	2	3	0.68	0.24		
	13		54	1:80	3.0	0	1	0.68	0.14		
14	6	Moderate		1:640	3.0	2	3	.07	0.31	3.75	
	12			1:2560	5.0	3	4	.31	0.75	2.63	
	22			1:640	2.0	3	3	.31	1.22	5.45	
15	4	Mild	67	1:640		3	4	0.14	0.38		
	17		42	1:640	4.0	0	0	0.34	0.82		
	66			1:40	3.0	0	1	0.07	0.20		
16	6	Mild	63	1:640	3.0	3	4	0.07	0.17	3.03	
	28			1:640	2.0	3	4	0.07	0.28	1.90	
17	3	Mild	48			3	4	0.10	0.61		
	4			Under 1:5	1.0	2	4				
	8		69	1:5	1.0	2	3	0.03	0.27		
	20			1:10	1.5			0.10	0.54	1.30	
18	4	Mild	50	1:640	1.5	0	0	0.10	0.48	2.78	
	8			1:160	3.0	0	0	0.07	0.34		
19	10	Mild		1:1280	4.5	0	2	0.27	0.68		

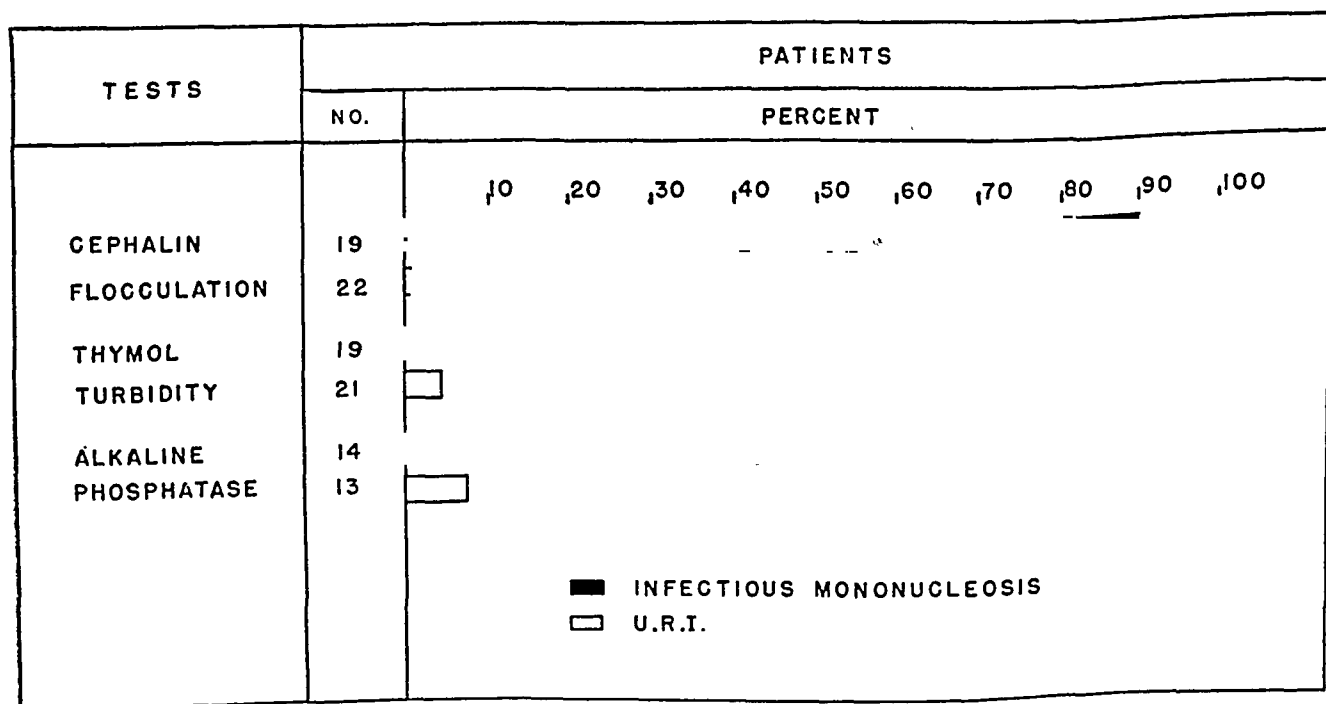


FIG. 1. PERCENTAGE OF PATIENTS WITH INFECTIOUS MONONUCLEOSIS WHOSE SERA SHOWED ABNORMALITIES IN LIVER FUNCTION TESTS AS COMPARED WITH PATIENTS WITH UPPER RESPIRATORY INFECTIONS. NO SERUM FROM A PATIENT WITH AN U.R.I. SHOWED AN ABNORMAL CEPHALIN FLOCCULATION TEST

TABLE II

Results of electrophoretic analysis of serum proteins from 2 convalescent infectious mononucleosis cases expressed as per cent of total protein

Condition	Albumin	Globulin		
		Alpha	Beta	Gamma
Inf. mononucleosis with jaundice	52.5	4.1	22.7	20.7
Inf. mononucleosis without jaundice	47.0	4.9	27.3	20.7
Normal	65-70	5-8	11-13	7-11

DISCUSSION

It has been pointed out in this paper that a demonstrable hepatitis occurs in many cases of infectious mononucleosis without jaundice. These results are in accord with previous investigations (1, 2). In addition the results of this study suggest that the cephalin-cholesterol flocculation reaction is a more sensitive indicator of this hepatic dysfunction than the thymol turbidity test and may be used in differentiating such cases from uncomplicated upper respiratory infections.

Histopathological evidence of liver involvement in non-jaundiced infectious mononucleosis cases has been reported from both punch biopsy studies (8, 9) and from autopsy reports (10 to 13). These consist of infiltrative mononuclear lesions frequently associated with destruction and disappearance of the liver cells, and the microscopic picture has been said to resemble early infectious hepatitis (8 to 10). Similar morphological studies carried out on the livers of patients with infectious mononucleosis complicated by jaundice (14, 15) have also demonstrated lesions closely comparable to infectious hepatitis.

The similarities between the hepatic involvement in infectious mononucleosis and infectious hepatitis are frequently reflected in the clinical picture of these diseases. Indeed, cases of infectious mononucleosis with jaundice frequently cause much difficulty in differential diagnosis from cases of infectious hepatitis (16). The hematological picture of the early phase of both naturally occurring (17 to 20) and experimentally induced (21) infectious hepatitis may closely simulate that of infectious mononucleosis and as many as 60 per cent of the lymphocytes have been described as atypical in infectious hepatitis (18). Increases of the characteristic heterophile antibody of infec-

tious mononucleosis have not been described in infectious hepatitis (18), although Eaton and his associates (22) have found a heterogenous antibody in a few such cases that could be differentiated only by absorption tests. Since increases of the heterophile antibody may not always be demonstrable in the serum of patients with infectious mononucleosis, occasional cases of these 2 diseases may be nearly indistinguishable.

SUMMARY AND CONCLUSIONS

1. The results of serial liver function tests carried out in this laboratory on 19 cases of infectious mononucleosis without jaundice are presented and indicate that demonstrable hepatic impairment occurs in many such cases.
2. The cephalin-cholesterol flocculation test may be a more sensitive indicator of this disturbance than the thymol turbidity test.
3. Use of the cephalin-cholesterol flocculation and the thymol turbidity tests may be helpful in distinguishing infectious mononucleosis from uncomplicated upper respiratory infections.
4. Relations between infectious mononucleosis and infectious hepatitis are briefly discussed.

Since the completion of this paper, the results of a similar study have been published by Q. B. DeMarsh and H. L. Alt (23) reporting abnormalities in the cephalin-cholesterol flocculation and/or the sulf-bromophthalein tests in 19 cases of infectious mononucleosis without clinical jaundice on whom serial liver function tests were performed. There has also been a report by Gall (24) recording similar findings in 34 cases.

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STUDY OF THE DISAPPEARANCE OF CONGO RED FROM THE BLOOD OF NON-AMYLOID SUBJECTS AND PATIENTS WITH AMYLOIDOSIS

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In a case suspected clinically of having amyloidosis because of the presence of a chronic infection, large liver and spleen, and moderate albuminuria, a Congo Red test done by the current standard method (1) proved to be negative according to present interpretation (60 per cent of the dye was removed from the blood at the end of 60 minutes). A moderate amount of amyloid was, however, shown to be present by biopsy of the liver. For this and other considerations it was decided to review the various methods of carrying out the test and the criteria employed in interpretation.

In 1923 Bennhold (2) described a method for the laboratory diagnosis of amyloidosis by the intravenous injection of 10 cc. of a 1 per cent solution of Congo Red.¹ He regarded the disappearance of more than 60 per cent of the Congo Red at the end of an hour as presumptive evidence of amyloid disease. Subsequent investigators (Friedman and Auerbach [1], Taran and Eckstein [3], Lipstein [4], Auerbach and Stemmerman [5]), modified Bennhold's original method because they found that many falsely positive results were obtained using his criteria. They suggested that at least 90 per cent of the dye be absorbed in an hour before making the diagnosis of amyloidosis.

In 1942, Taran and Eckstein (3) found that the 4-minute specimen was frequently too light in color to be relied upon as the standard or 100 per cent specimen. They recommended that a 2-minute specimen be taken in addition to the 4-minute and 1-hour samples, advising that the 2-minute specimen be used as the standard for comparison (100 per cent). They gave 1 cc. of a 1 per cent Congo Red solution for each 10 pounds of body weight. In order to avoid errors due to

hemolysis, they precipitated the liberated hemoglobin with acetone. Some time earlier, Friedman and Auerbach (1) had used 95 per cent alcohol as the hemoglobin-precipitating agent.

Harmon and Kernwein (6) in 1942 modified the Congo Red test by using known standard solutions of Congo Red for comparison. They used isotonic sodium oxalate solution as the anti-coagulant, performing the determinations on plasma. They tried to avoid hemolysis by careful collection of specimens rather than by correcting for it by precipitation of the hemoglobin. It should be noted, however, that they also used a 4-minute specimen as their 100 per cent standard.

There are at least 3 significant sources of error in all the methods previously described. The first lies in the assumption that the injected dye is completely mixed at the end of 2 or 4 minutes. That this is not true for most persons has been amply demonstrated by Gregersen (7), Gibson and Evans (8), Price and Longmire (9). Using Evans blue, these investigators showed that at least $7\frac{1}{2}$ to 20 minutes are required for complete mixing in normal people and even longer in persons with slower circulations. The second important source of error lies in the assumption that little or no absorption of the injected Congo Red takes place in amyloidosis before the 2- or 4-minute specimen is obtained. Evidence presented in this paper suggests that this assumption may well be the main source of error in the so-called "false-negative" tests (Figure 1). The third source of error is the personal equation which enters into the color matchings whenever optical colorimeters are used.

In an effort to estimate the magnitude of these errors and correct for them, it was decided to study the behavior of intravenously administered Congo Red by determining dye concentration in the

¹ The Congo Red used in this study was furnished by the C. F. Kirk Co., New York City.

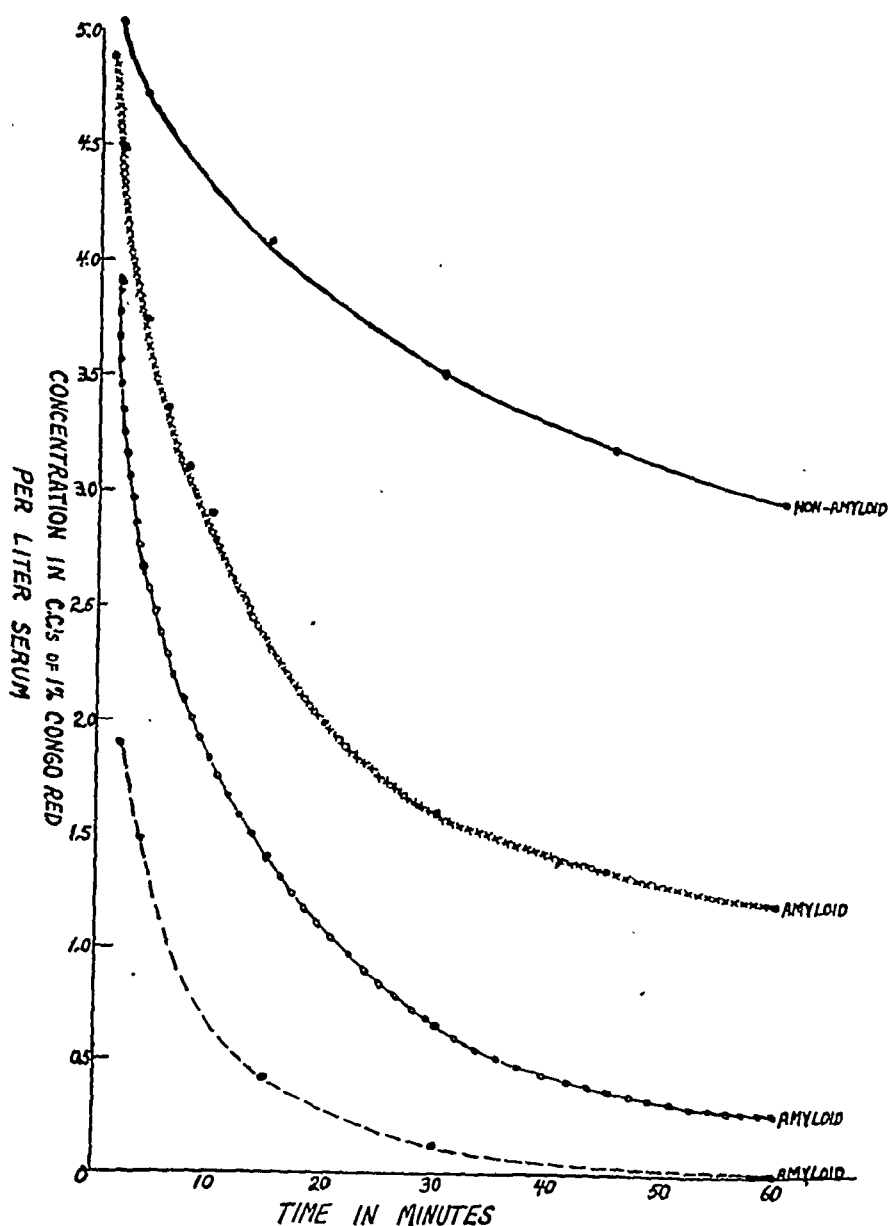


FIG. 1. DISAPPEARANCE CURVES IN NON-AMYLOID AND AMYLOID SUBJECTS

serum at frequent intervals during the course of an hour after its administration. The urine was also collected during this hour and Congo Red, if present, was quantitatively assayed. All determinations were done on the Coleman spectrophotometer. The rate of disappearance of Congo Red from the blood stream in normal persons as well as in persons with amyloid disease was studied.

Because neither a 2- nor 4-minute specimen appeared to be reliable for use as the 100 per cent standard, initial effort was directed toward finding a method for determining the theoretical concentration of Congo Red in the blood following

intravenous injection of a known amount. Several means were open to study.

One was obviously the determination of plasma volumes, which would at once permit calculation of the theoretical concentration of Congo Red at zero time with complete mixing. Using T-1824 (Evans blue) in non-amyloid subjects, satisfactory measurements of blood volume were obtained. In patients with amyloid disease, however, abnormally high plasma volumes were found. For example, in patients who weighed 90 to 110 pounds, plasma volumes of 4100 to 5200 cc. were obtained. One explanation was that since Evans blue is a vital dye, it was likely that some of it had been

fixed by amyloid tissue. On staining amyloid tissue obtained at post-mortem examination with Evans blue, it was found that such was the case. Much more of the dye is fixed by this tissue than by normal tissue.

A rough method for determining plasma volume is the one based on surface area or body weight. From previous reports (8, 10, 11, 12) this is about 44 cc. per kilogram body weight, or 1628 cc. per square meter surface area. However, these

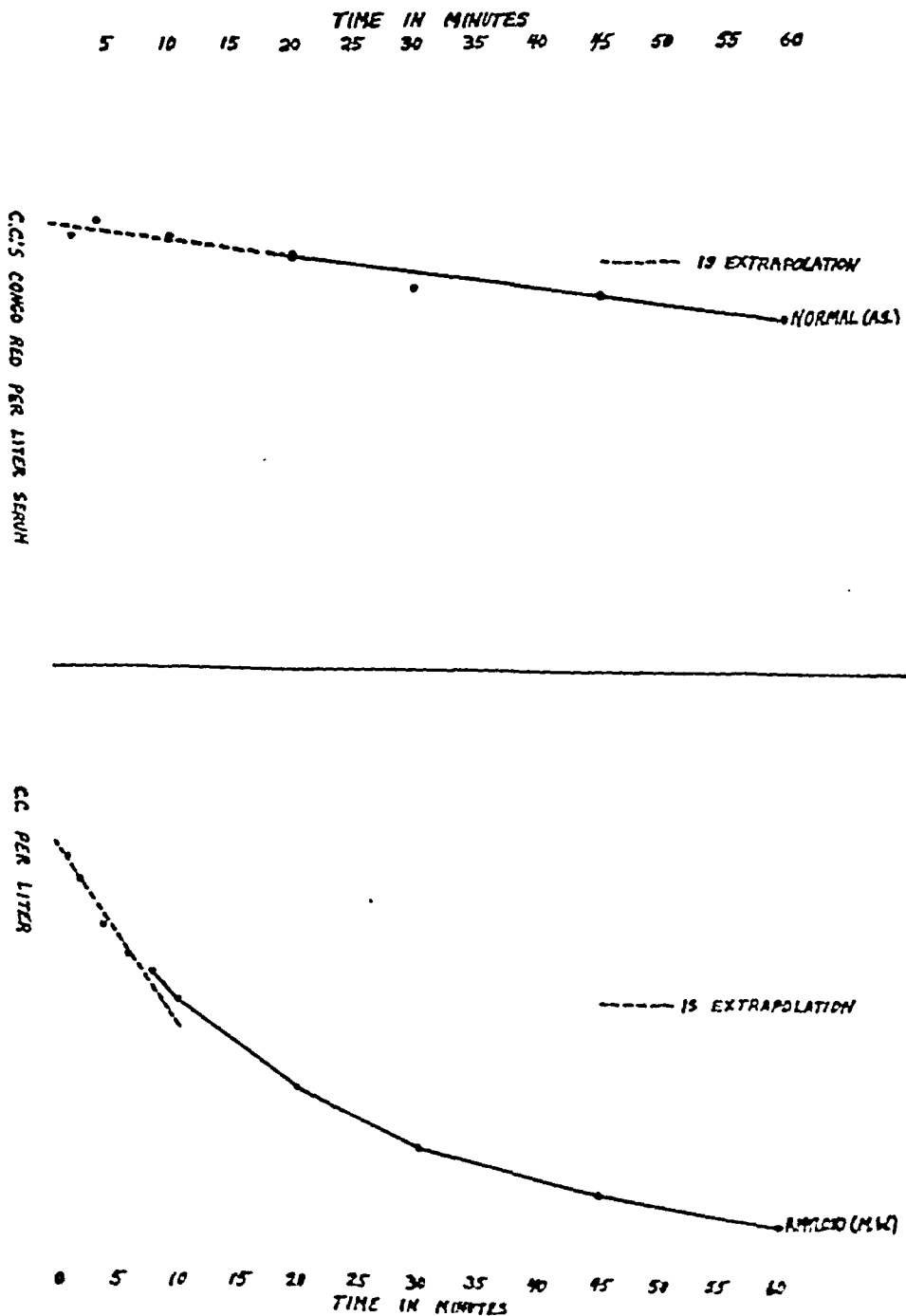


FIG. 2. EXTRAPOLATION OF C_0

figures represent average values for normal individuals between the ages of 17 and 89 and variations that occur in disease may introduce significant errors. A third method for measuring plasma volume is by introduction of radioactive material in the form of red blood cells, but this means was not at the moment available.

An alternate method for determining the theoretical concentration of Congo Red is the extrapolation to zero time of a curve which is plotted from a large number of determinations. In normal persons it was found, in an effort to determine the theoretical concentration, that a straight line is obtained when points between 10 and 60 minutes are plotted. Points between 2 and 10 minutes are variable and consequently unreliable because mixing is incomplete.

In amyloid disease, however, the curve on semilogarithmic paper was not straight. The theoretical concentration could best be approximated by extrapolation derived from that portion of the curve obtained from values in the first 10 minutes. The curve in this portion was more nearly straight probably because rapid disappearance of the dye tended to mask the vagaries of mixing (Figure 2).

METHOD

1. Preparation of standard Congo Red absorption curve.

1 cc. of a 1 per cent Congo Red solution is diluted to 500 cc. with a 1:1 acetone water solution. This is used as a stock solution. Solutions of 10, 25, 37½, 50 and 75 per cent are prepared and their transmission value, determined in a Coleman Junior Spectrophotometer, is plotted against concentration on semilogarithmic paper. This yields a straight-line relationship up to 75 per cent concentration. Beyond this point there is a slight change in the slope of the line. By diluting the unknown specimens, one can keep within the 10 to 75 per cent range without difficulty. It was found that identical readings with the acetone water diluent were given by using distilled water alone as the diluting agent in the preparation of the standard. A wave length of 500 was found to be the most satisfactory, and this is the one generally recommended. It is important to prepare the standard and perform the tests with the same lot of Congo Red, because of the known variations in different batches of organic dyes.

2. *Technique of the test.* The Congo Red determination is done after a fast of at least 8 to 12 hours to avoid the lipemia which interferes with colorimetric readings. A blood specimen to serve as the blank control is taken prior to the injection of 1 cc. of 1 per cent Congo Red solution per 10 pounds of body weight. 10 cc. blood samples are removed from the opposite arm at 2, 4, 6, 8,

10, 20, 30, 45 and 60 minutes. The needle may remain in place for the withdrawal of the first 5 specimens without using anti-coagulants.

The blood is permitted to stand at room temperature for 1 hour and then placed in the refrigerator for a half hour to facilitate separation of the clot. The serum is separated by centrifugation and 2-cc. amounts are placed in centrifuge tubes. Depending upon the intensity of color, gauged by the naked eye, 1:4 dilutions with acetone at room temperature are prepared for the stronger solutions, and 1:2 dilutions for the weaker ones. The blank is treated in an identical manner. All of the serum specimens from a given patient are treated in the same way.

After the addition of the acetone, the tubes are stoppered, vigorously shaken to insure complete mixing and thorough precipitation of the hemoglobin and protein. The specimens are then centrifuged for 10 minutes at 10,000 r.p.m. to sediment the precipitate. A crystal-clear solution of Congo Red is regularly the result.

Serum rather than plasma was used for 3 reasons. First, because the use of 50 mgm. of dry sodium oxalate per 100 cc. of whole blood significantly changes the hematocrit reading by shrinking the RBCs (13). Second, isosmotic sodium oxalate would introduce a source of error by dilution in the concentration of the dye. Third, the use of heparin introduces a yellow color into the plasma.

That the process of clotting does not remove a significant amount of dye from the serum is shown by comparing the dye concentration in serum and plasma prepared from the same whole blood specimens.

Concentration in serum	Concentration in plasma
3.88	3.32
3.72	3.36
3.52	3.36
3.36	3.20
3.20	2.92

Dry sodium oxalate was used as the anti-coagulant, and the resulting shrinkage of the RBCs and consequent increase in plasma volume account for the lower dye concentration in the plasma compared with the serum. Keith, Rowntree and Geraghty (14) showed that the RBCs do not take up any of the vital dyes.

Nor does the precipitation of serum proteins and hemoglobin resulting from hemolysis remove any dye. This was demonstrated by quantitative recovery of known amounts of added dye from serum and plasma solutions after protein precipitation.

The supernatant fluid is decanted into colorimetric tubes and the colorimeter set at 100 with the blank in position. Readings are then taken at a wave length of 500. It is quite safe to allow the serum to stand in stoppered tubes in a refrigerator for several days without any alteration of the color intensity as determined by the spectrophotometer.

3. *Calculation of results.* Results are expressed in the form of cc. of 1 per cent Congo Red per liter of serum. The transmission values of the various specimens are converted into concentration by the prepared curve of

standard dilutions. These values are then multiplied by the dilution used in preparing the protein-free solution.

Example: The colorimeter reading in per cent transmission is 43. The concentration obtained from the standard dilution curve is 1.34 cc. per liter. Since a 1:4 dilution was made in preparing the protein filtrate, the concentration of Congo Red in the serum is, therefore, 5.36 cc. of 1 per cent Congo Red per liter.

Excretion of dye in urine

Some investigators have stated (6), and it is a current concept that under certain conditions, intravenous injection of Congo Red may be followed by the appearance of the dye in the urine. When this occurs, the test is supposed to lose value. To investigate the validity of these statements, a method for the extraction and quantitative estimation of Congo Red in the urine was worked out.²

An extracting reagent is prepared by making a 1 per cent solution of quinine sulphate in chloroform or ethylene dichloride. This solution must be freshly prepared because rapid discoloration occurs. This is filtered and 10 cc. of 1 N NaOH is added to each 100 cc. of solution. To 30 cc. of urine, 5 cc. of saturated NaHCO_3 is added, to which, in turn, is added 35 cc. of the extracting reagent. The mixture is vigorously shaken and then centrifuged. The process is repeated until all of the dye has gone into the extracting solution. The supernatant layer is then aspirated. To 10 cc. of the quinine Congo Red solution 3 cc. of 1 N NaOH is then added to bring the pH of the solution to 8 or 8.5, at which pH the dye goes into the aqueous phase when the mixture is vigorously shaken and centrifuged. The colored solution is then read in the spectrophotometer at a wave length of 500 against a urine blank and a distilled water blank.

It has been found that this method is accurate enough to detect 1/10,000 cc. of Congo Red per 100 cc. of urine. For ready comparison, a series of standards with known concentrations of the dye can be set up. In the series of Congo Red determinations reported in this paper, no urine was found which contained more than a faint trace of dye, despite the presence of large amounts of albumin, too little to affect the concentration in the blood.

Characteristics of the disappearance curve

It is apparent that in the normal subject 2 mechanisms are operating; namely, mixing, which occurs in the first 8 to 15 minutes, and removal of the dye from the blood stream presumably by the reticulo-endothelial system (Figure 2). In the presence of amyloid, the mixing factor is obscured by the very rapid removal of the dye during the first 30 minutes after injection (Figure 2). Removal during the second 30 minutes is much slower. It is presumed that this initial phase of rapid removal is referable to fixation of the injected dye in the amyloid

tissue. In the second 30 minutes, the slower rate of removal approaches the disappearance rate occurring in normal individuals.

The greatest differences between the curves of normal individuals and those with deposits of amyloid occur in the first 30 minutes. It would be reasonable, therefore, to base a Congo Red test on this time interval. Results can be estimated in 2 ways. One, serum concentration of dye is plotted against time on semi-logarithmic paper, and the resulting line is extrapolated back to zero time (Figure 2). The concentration obtained at zero time is used as the standard with which to compare the value at 30 minutes, and the percentage of dye which has been absorbed or lost from the blood stream is calculated. A simpler but somewhat less accurate method is to calculate the concentration of dye at zero time on the basis of a serum volume deduced from body weight or surface area relationships.

The plasma or serum volume in normal individuals ranges from 1624 to 1680 cc. per square meter of surface area with an average value of 1628, and 40 to 49 cc. per kilogram, with an average of 44 (8, 10, 11, 12). In sthenic individuals the larger values are used. The C_0 (concentration at zero time) is compared with a 30-minute specimen as previously noted. The advantage of this method is that it avoids numerous venipunctures and processing of numerous blood specimens. Evidently it cannot be applied to patients with cardiac failure, dehydration or other situations where a significant deviation of the plasma volume from the normal is to be expected.

In this study, the C_0 was obtained by the extrapolation of curves of concentration. However, it was found that the quantities of Congo Red absorbed after 30 minutes, calculated from extrapolation of curves to zero time or estimated on body weight or surface area, were so nearly equal in value that for practical purposes the simpler method might be used. The validity of this statement needs to be verified by a larger number of tests in both normal and amyloid subjects than is now presented by the table below comparing values obtained from extrapolation and estimation in normal and amyloid subjects.

Patient	Surface area in sq. meters	C_0 calculated	C_0 extrapolated	Per cent removed in 30 min.	
				Calc. C_0	Extrap. C_0
B. R.	1.88	5.70	5.75	14	15
K. Z.	1.94	5.50	5.40	13	12
M. Z.	1.87	5.25	5.15	15	13
M. M.	1.58	5.20	5.70	65	71
M. W.	1.42	5.90	5.20	73	69
J. M.	1.67	4.90	4.00	87	84

RESULTS

Using the method outlined, the disappearance of Congo Red from the blood was studied in 74 subjects, 10 of whom were healthy young adults, 52 with rheumatoid arthritis, and the remaining

²We are indebted to Dr. B. B. Brodie, N. Y. U. Research Service, Goldwater Memorial Hospital, for developing these procedures.

12 with various diseases. The results are given in terms of percentage of dye removed from the blood in 30 minutes (Figure 3). Sixty-eight cases showed from 4 per cent to 32 per cent removal of injected dye in 30 minutes. None of these patients had any clinical evidence of amyloidosis. In the remaining 6 patients, all with rheumatoid arthritis, 68 per cent to 96 per cent of the injected dye was removed from the blood in 30 minutes. All of these patients showed confirmatory clinical findings of amyloidosis. The diagnosis was verified by liver biopsy in 2 patients where consent to the procedure was given. One had a Congo Red absorption of 69 per cent and the other 96 per cent.

In the subjects with amyloidosis, Congo Red was not found in the urine. In only 5 of the other subjects did Congo Red appear in the urine, and then in such small amounts as to be insignificant from a quantitative point of view.

A frequency distribution curve of the amounts of dye removed from the blood in 30 minutes shows that the patients with evidence of amyloidosis fall in an entirely different segment from non-amyloid subjects (Figure 4). Of interest is the fact that

1 of the patients with amyloid disease showed, on liver biopsy, deposition of amyloid in only several small blood vessels. He absorbed only 69 per cent of the dye at the end of 30 minutes.

The range of values in non-amyloids at 30 minutes varies from 4 per cent to 32 per cent removal, with an average of 17 per cent (Figure 3). This compares with a range of 15 per cent to 51 per cent and an average of 31 per cent for this same group at 60 minutes.

In the amyloids, the range of values at 30 minutes is from 68 to 96, with an average of 80 per cent. At 60 minutes the range is 77 to 100 with an average of 88 per cent. The difference of 8 per cent between the average values at 60 and 30 minutes in amyloids supports the concept that the removal of dye by amyloid tissue occurs largely in the first 30 minutes.

The data presented here indicate that the 2- or 4-minute blood specimen may be too dark (because of incomplete mixing) or too light in color (because of rapid fixation by amyloid) to be reliable as a 100 per cent standard for comparison. A better value is obtained from extrapolation of

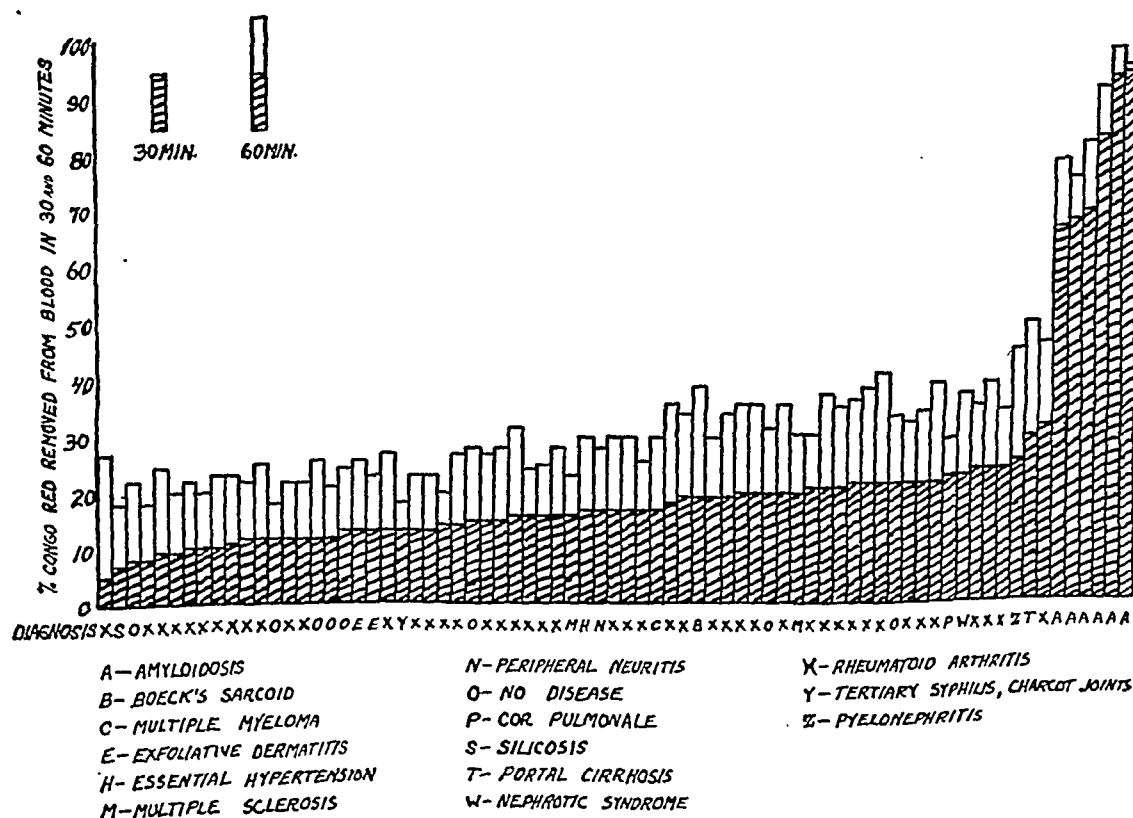


FIG. 3. DISAPPEARANCE OF CONGO RED FROM BLOOD STUDIED IN 74 SUBJECTS
Results given in terms of percentage of dye removed in 30 minutes

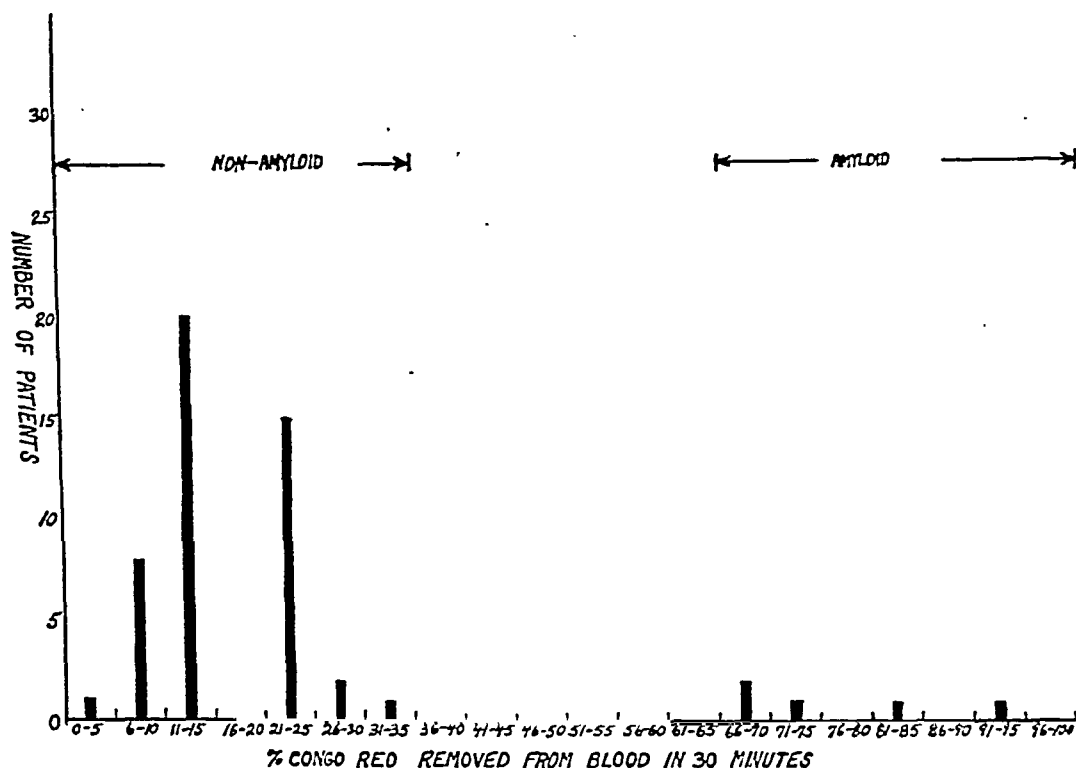


FIG. 4. CONGO RED REMOVED FROM BLOOD IN 30 MINUTES IN NON-AMYLOIDS AND AMYLOIDS

the curve of disappearance or calculation of blood volume from surface area or body weight. Because the greatest difference in the curves of removal of Congo Red from the blood of non-amyloid and amyloid patients exists at about 30 minutes after injection, this time interval seems the one of choice as the end point of the test. Since the normal range extended as high as 32 per cent removal of the dye from the blood at the end of 30 minutes, more than 35 per cent removal in this time interval is presumptive evidence of amyloidosis. However, a larger series of normal persons is needed before the critical level is decided upon.

CONCLUSION

1. The use of Congo Red for testing for the presence of amyloidosis has been simplified and the accuracy increased by

- calculating the theoretical initial concentration rather than using a 2- or 4-minute specimen for comparison,
- using 30 minutes rather than an hour as the end point.

2. T-1824 (Evans Blue) cannot be used for the calculation of blood volume in amyloidosis, because amyloid tissue fixes this dye also.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF
HUMAN PLASMA FRACTIONATION. XXXIV. COMPARATIVE STUDIES
ON THE NUTRITIVE VALUE OF ORALLY AND INTRAVENOUSLY
ADMINISTERED HUMAN SERUM ALBUMIN IN MAN^{1, 2, 3}

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Human serum albumin for therapeutic use was developed and produced during the war as a colloid of high osmotic properties, chiefly for the treatment of shock. Its usefulness in this regard has been attested by several workers (1 to 3). In addition, the use of concentrated albumin solutions has been studied in chronic Bright's disease (1, 4), and in cirrhosis of the liver (1, 5). In both these conditions therapy was directed at increasing the serum albumin concentration and promoting diuresis.

When a protein is administered either orally or parenterally as a partial or a complete source of nitrogen for an individual, care must be taken that its nutritional adequacy is known. In this regard there are 2 important considerations: (1) Does the protein contain all the essential amino acids in optimal amounts; and (2) is the protein readily available for the metabolic needs of the body?

In answer to the first, human serum albumin has been shown to contain a low concentration of tryptophane (6), and, in rat growth experiments, fortification with both tryptophane and isoleucine was required to obtain optimum growth (7). No

supplementation was required for the dog. These studies indicate that human albumin may be deficient in 2 of the amino acids essential to man (8).

As for the second question, the ready availability for metabolism of parenterally administered plasma proteins has been the subject of discussion and recent investigation. There have been observations by several investigators that following the intravenous injection of whole protein (plasma protein, whole blood and plasma albumin) there is a rise in plasma protein, a minimum excretion of nitrogen in the urine, and a positive nitrogen balance. This would suggest that whole protein parenterally administered was not as rapidly metabolized as whole protein given by mouth or hydrolyzed protein by vein. Thus, a rise of the plasma albumin concentration in patients with cirrhosis of the liver immediately following the administration of human albumin intravenously was observed by Thorn *et al.* (5). Browne *et al.* (9) found intravenously administered plasma would maintain a positive nitrogen balance in the "catabolic period" after damage in man, but that a protein hydrolysate given in similar amounts was almost quantitatively excreted as urea nitrogen. Elman and Davey (10) observed that plasma transfusions would correct the hypoalbuminemia of protein-depleted dogs, although in the period following this therapy the temporarily elevated plasma albumin dropped to its previous low level and much of the plasma protein nitrogen retained appeared in the urine. Albright (11) has shown that following the intravenous administration of plasma protein, "burning" and "conversion" of the protein did not start to an appreciable degree until at least the third post-injection day (at which time urinary nitrogen excretion increased and urinary phosphorus and potassium excretions decreased), and that the nitrogen excretion from the injected plasma was not

¹ This is paper No. 57 in the series, "Studies on Plasma Proteins," from the Harvard Medical School, Boston, Mass., on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

² This study was aided in part by a grant from the Abbott Laboratories, North Chicago, Ill.

³ The albumin administered intravenously was obtained in part from the Bureau of Medicine and Surgery, U. S. Navy Department, and in part was specially prepared by the Plasma Fractionation Laboratory, Harvard Medical School, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The albumin administered orally was supplied by the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

complete in the subsequent 1 to 3 weeks. Approximately 50 per cent of the injected plasma protein was eventually burned and the remainder converted into tissue protein. Whole blood and plasma albumin behaved in a manner similar to blood plasma. During plasma transfusions in man, Meyer *et al.* (12) noted a decrease in the urinary nitrogen excretion which resulted in a marked positive nitrogen balance, but 13 per cent, 87 per cent, and 50 per cent of the "retained" nitrogen were subsequently excreted in the urine. However, Whipple and co-workers (13) have never observed a significantly increased excretion of urinary nitrogen following plasma infusions in dogs, even when the dogs were rendered diabetic with phlorhizin (14).

In an attempt to answer the questions of the nutritional adequacy and availability of intravenously and orally administered albumin in man, the studies reported here were made. Five healthy young males in good nutritional status were placed on a diet almost devoid of protein, but thought to be adequate in calories and vitamins. Protein was supplied either orally as lactalbumin⁴ or human albumin, or intravenously as partially hydrolyzed (enzymatic) casein⁵ or as human albumin solu-

tion. The actual volume of each of the solutions and also the amount of powdered lactalbumin to be administered were determined from the nitrogen content by macro-Kjeldahl analysis of each. Daily nitrogen balance was followed. Hematocrit, plasma volume, and plasma protein concentration were determined at suitable intervals.

METHODS

A. Protein-free diet

A diet designed to provide from 3000 to 3300 calories daily with a minimum nitrogen intake was modified from that used by Rose (8). The diet is presented in Table I.⁶ Samples of each food used in this diet were analyzed by the macro-Kjeldahl procedure and it was found that the total nitrogen contained in the daily food intake was 0.3 gram for the first 2 individuals and 0.1 gram for the final 3. A salt mixture was not provided, nor were vitamins which were not included in the vitamin capsule. We do not believe that these deficiencies were serious, except perhaps for the salt loss which probably took place during the periods of diarrhea in 2 individuals who took albumin orally. Nitrogen equilibrium or slight positive balance and constant weight have been obtained with this diet using purified casein (Labco) as the sole source of nitrogen.

⁶ The authors wish to thank Mrs. Elizabeth Caso, Instructor in Nutrition, Harvard School of Public Health, for advice in preparing this diet, and Miss Florence A. Packman, Director of Home Economics of the Spry Kitchen of Lever Brothers Company, Cambridge, Massachusetts, for developing and testing the recipe used for the cornstarch cookies.

TABLE I

	Carbohydrate	Nitrogen	Fat
	<i>grams</i>	<i>grams</i>	<i>grams</i>
Cornstarch pudding	93.8	None	30.0
88 gm. hard candy	88.0	None	
400 cc. ginger ale	37.8	None	
119 gm. "beta lactose"	119.0	Not measured	
51 gm. centrifuged butter		0.001	51.0
6 cornstarch cookies	87.6	0.026	19.8
60 gm. currant jelly	39.6	0.016	
360 cc. coca cola	43.0	0.012	
300 cc. lemon juice*	29.4	0.190	
60 gm. canned pear (juice pack)	4.8	0.021	
1 "dayamin"† vitamin capsule		0.016	
Total	543.0	0.282	100.8
1.8 gm. protein and 3089 calories			

* In part of the study a synthetic lemon powder was substituted for the lemon juice, reducing the nitrogen intake by 0.19 gm.

† Furnished through the courtesy of Abbott Laboratories, North Chicago, Ill., and containing per capsule: Thiamine hydrochloride 5 mgm., riboflavin 5 mgm., nicotinamid 25 mgm., pantothenic acid 5 mgm., pyridoxine 1.5 mgm., ascorbic acid 100 mgm., vitamin A 10,000 U.S.P. units, vitamin D 1000 U.S.P. units.

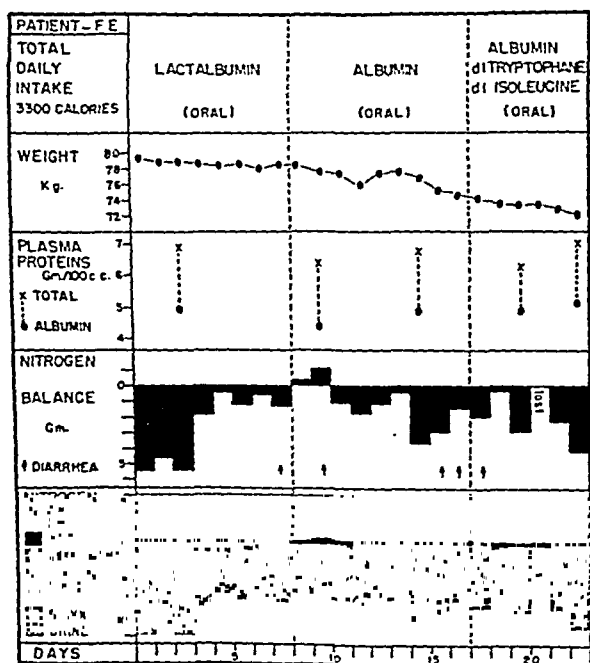


FIG. 1

B. Biochemical methods

The daily urine and pooled-stool nitrogen and total plasma protein were determined by micro- or macro-Kjeldahl digestion and subsequent nesslerization. Plasma albumin and globulin separations were made in part on the electrophoresis apparatus of Tiselius⁷ and in part by Howe precipitation (15). Blood volume measurements were made by the method described by Gregerson (16). Urinary tryptophane excretion was determined by the method of Shaw and McFarlane (17). Urinary alpha amino nitrogen determinations were by the gasometric ninhydrin method described by Van Slyke, MacFadyen and Hamilton (18).

RESULTS

The results will be divided into 3 parts; first, the 3 subjects to whom all protein was given by mouth; second, the 2 subjects to whom all protein was given intravenously; and third, the subject who received protein both orally and intravenously. There were no reactions to the intravenous administration of the albumin or the casein hydrolysate except once when the latter was given too rapidly and the individual became nauseated.

* The authors express their appreciation to Dr. S. Howard Armstrong, Jr., Department of Physical Chemistry, Harvard University, for these determinations and their interpretation.

I. Protein administered orally (Figures 1, 2 and 3)

Two subjects (F. E. and J. A.) were given during the first period lactalbumin orally to supply 37.5 grams of protein for 7 days in one, and for 8 days in the other. Albumin solution equivalent to 37.5 grams of protein was then substituted for the lactalbumin for 14 and 15 days, respectively, supplemented with 2.0 grams of dl-isoleucine⁸ and 2.0 grams of dl-tryptophane⁸ for the final 6 days. The daily protein intake was approximately 0.5 gram per kilogram of body weight. During the entire study of these subjects, both during lactalbumin and albumin administration, the excretion of nitrogen was greater than the intake by between 1.0 to 3.0 grams, except during the first 2 or 3 days when negative nitrogen balance was more marked, reflecting the previous high protein diet. When tryptophane and isoleucine were added to the diet during the period of albumin administration, there was no significant change in nitrogen excretion or balance. Both subjects noted weakness, had moderate intermittent diarrhea and nausea, and gradually lost weight (6 kgm.

⁸ Supplied by Merck & Co., Inc., Rahway, N. J.

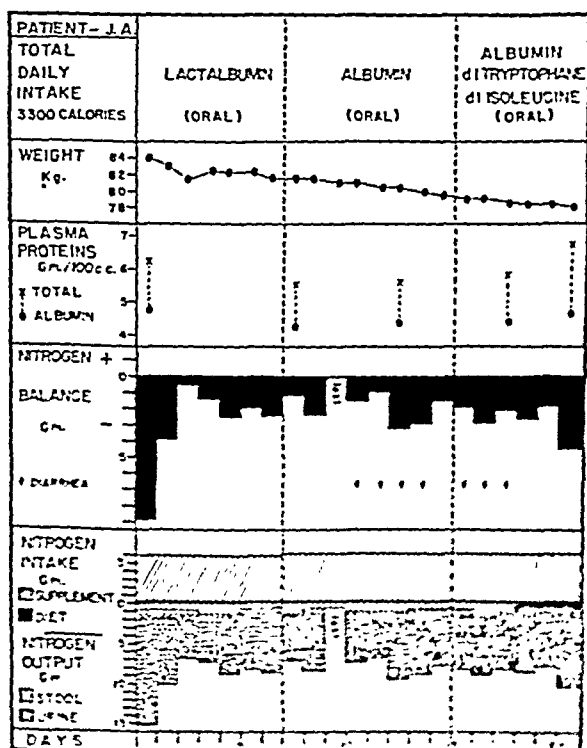


FIG. 2

TABLE II

Relation between protein administered as hydrolysate, lactalbumin, and albumin orally and intravenously and the nitrogen excreted

Period of study (days) and condition of study	1	2	3	4	5	6	7
	Total protein administered for period	Total nitrogen excreted for period	Total protein retained or lost	Minimum per cent protein retained in body* $\frac{(3)}{(1)} \times 100$	Total albumin increase in blood†	Per cent of protein administered retained in blood $\frac{(5)}{(1)} \times 100$	Per cent of protein retained found in blood $\frac{(5)}{(3)} \times 100$
F. E.	<i>grams</i>	<i>grams</i> ($\times 6.25$)	<i>grams</i>		<i>grams</i>		
	1-9 Lactalbumin orally	312	439	-127			
	9-24 Albumin orally	574	728	-154			
	Total		-281				
J. A.	1-8 Lactalbumin orally	273	405	-132			
	8-22 Albumin orally	535	727	-192			
	Total		-324				
W. C.	1-11 Albumin orally	533	426	+107			
W. K.	4-13 Protein hydrolysate intravenously	274	319	- 45			
	13-21 Albumin intravenously	271	216	+ 55	20	13	24
	Total		+ 10				
D. R.	4-13 Protein hydrolysate intravenously	274	322	- 48			
	13-29 Albumin intravenously	620	468	+152	25	70	46
	29-37 Protein hydrolysate intravenously	314	371	- 57			
	Total		+ 47				
W. C.	1-5 Protein hydrolysate intravenously	301	231	+ 70			
	5-9 Albumin intravenously	301	96	+205	68	86	42
	9-12 Albumin orally	226	185	+ 41			
	Total		+316				

* Assuming all nitrogen excreted came from administered albumin.

† Albumin concentrations (Table III, column 6.)

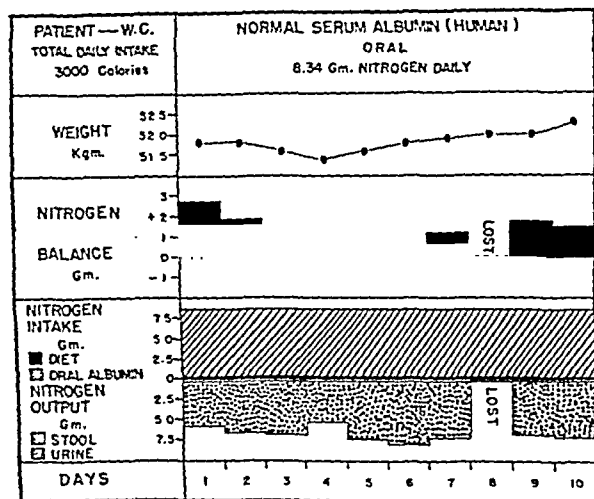


FIG. 3

and 7 kgm.) during the study, although both noted a prompt return of their sense of well-being, had normal bowel function, and regained much of their lost weight within 3 to 4 days after the study was discontinued and a normal diet was taken. Cumulative negative nitrogen balances for these subjects represent about 280 and 325 grams ($N \times 6.25$) protein loss from the body tissues (Table II, column 3). There was no significant change in plasma albumin concentration or hematocrit except during periods of probable mild dehydration associated with diarrhea.

In contrast to the 2 individuals just cited, the third (W. C.) received 50.0 grams of normal human serum albumin orally daily as the sole source of nitrogen. He remained in slight positive nitrogen balance throughout, gained slightly in weight, and felt entirely well during the 10-day study period. No tryptophane or isoleucine supplements were given.

II. Protein administered entirely intravenously (Figures 4 and 5, Table III)

Two subjects (W. K. and D. R.) were also given 37.5 grams of protein daily, this time intravenously in all experimental periods, again in amounts of about 0.5 gram per kilogram of body weight. Casein hydrolysate was given during the first period of 9 days, followed by human serum albumin for 16 days in one, and only 8 days in the other subject who became ill so that the study was discontinued (acute prostatitis). The sub-

ject who continued for 16 days on albumin was given intravenously 2.0 grams of dl-tryptophane and 2.0 grams of dl-isoleucine with the albumin during the last 6 days, and followed by a final period of 37.5 grams of the casein hydrolysate daily for 8 days.

After a brief period of negative nitrogen balance on the protein-free diet, intravenous casein hydrolysate administration was begun and approximate nitrogen equilibrium was soon achieved. A positive nitrogen balance of significant degree was obtained (except for occasional days) during the 8 and 9 days of the administration of unsupplemented albumin. The individual (D. R.) who continued albumin administration, this time supplemented with tryptophane and isoleucine, continued to exhibit a positive nitrogen balance, although in this instance perhaps somewhat less marked. Slight but persistent negative nitrogen balance was obtained in this subject during the final 8-day casein hydrolysate period. After this period, the subject ate very well of a normal diet containing at least 100 grams of protein daily.

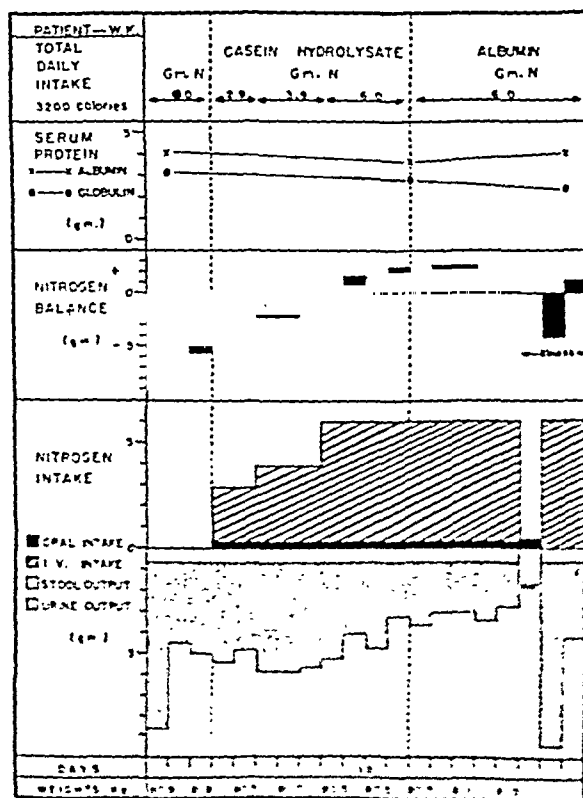


FIG. 4

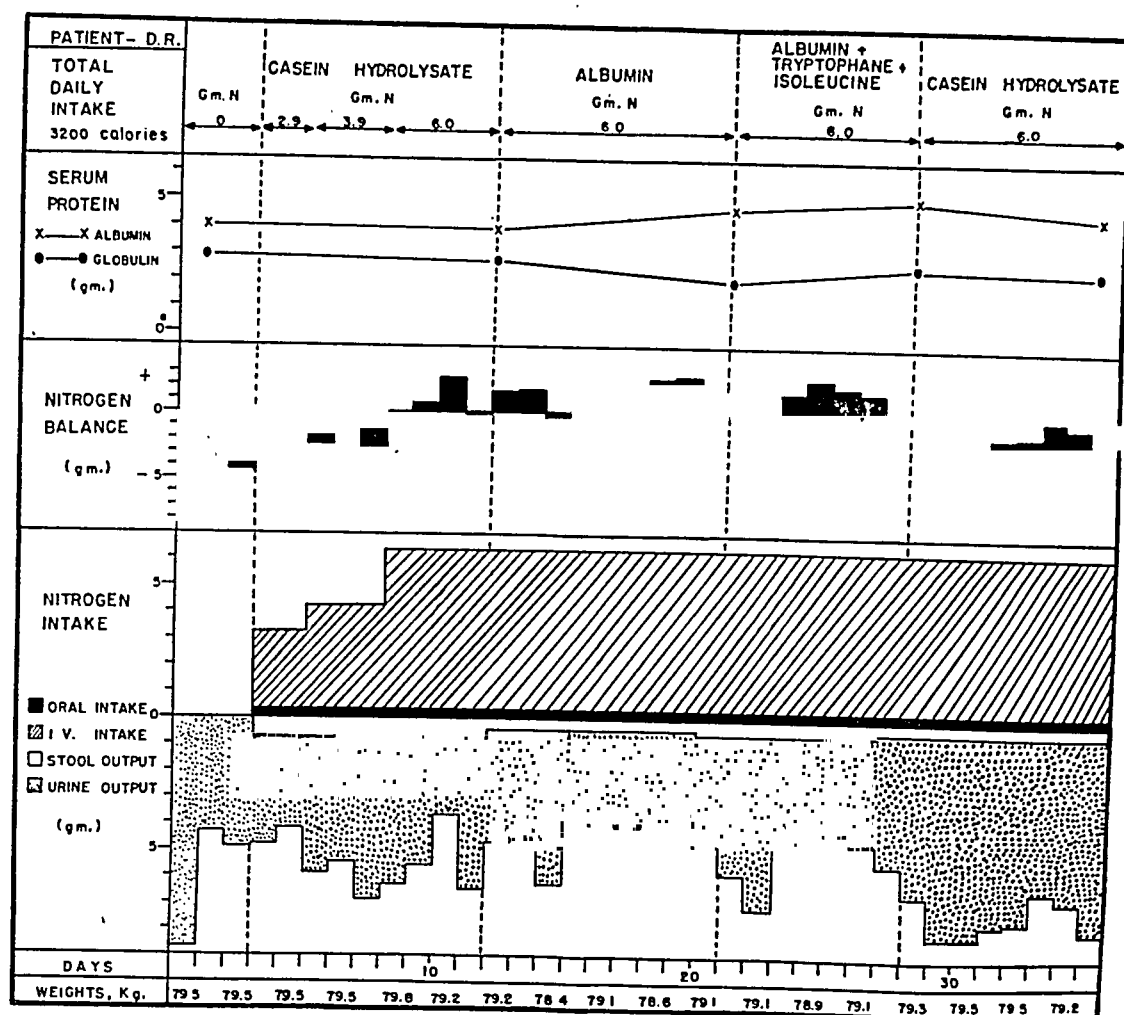


FIG. 5

The plasma albumin concentration remained the same in the individual (W. K.) given only 8 days of albumin, but rose progressively in the other during the 16 days of albumin administration, and subsequently fell to the control value by 24 days after albumin administration was discontinued.

An increase in plasma volume was observed after both the administration of casein hydrolysate and albumin (Table III, column 4). This is most clearly seen in subject D. R., in whom the plasma volume decreased again somewhat after the normal diet had been resumed. Hematocrit determinations in both decreased as the blood volume rose. This was most evident in D. R. at the end of the period of albumin administration.

There was an increase in the total circulating albumin during the period of albumin administration which was slight in subject W. K., but was striking in subject D. R. (Table III, column 5). In Figure 6 are plotted the values in this subject for the total increase or retention of albumin in

the plasma determined at the conclusion of the albumin administration and the decreasing values at intervals thereafter (Table III, column 6). These values fall on a 6-day 50 per cent disappearance curve, calculated from the general equation of decay.⁹ In this subject, therefore, one-half of the plasma albumin retained during the period of albumin administration had disappeared from the plasma in 6 days, one-half of the remaining "extra" albumin during the next 6 days, et cetera,

⁹ General Equation of Decay: $A = A_0 e^{-kt}$, where A = quantity of albumin retained in the plasma at any time, t , following the cessation of intravenous albumin infusions; A_0 = quantity of albumin retained in the plasma at the conclusion of the albumin administration; t = time in days after cessation of infusions; $k = \frac{0.6932}{t} = 0.1733$ (50 per cent decay in 4 days), 0.1386 (50 per cent decay in 5 days), and 0.1155 (50 per cent decay in 6 days). The values are plotted in Figure 6 as Decay Coefficients $\left(\frac{A}{A_0}\right)$ by rearranging the formula so that $\frac{A}{A_0} = e^{-kt}$.

TABLE III

Plasma volume and protein values in three subjects given albumin intravenously

Day of study and condition of study		1	2	3	4	5	6
		Total plasma protein concentration	Plasma albumin concentration*	Hematocrit	Plasma volume	Total circulating plasma albumin	Total albumin increase in plasma
		grams per 100 cc.	grams per 100 cc.	per cent	cc.	grams	grams
W. K.	1 Control	7.04	4.01	49.4	3620	145	
	13 After 9 days protein hydrolysate intravenously	6.03	3.44	45.7	4199†	144	0
	21 After 8 days albumin intravenously	6.37	4.01	42.9	3977	157	+13
D. R.	1 Control	6.88	3.99	48.0	3224	129	
	13 After 9 days protein hydrolysate intravenously	6.54	3.86	47.5	3857	149	0
	22 After 9 days albumin intravenously	6.61	4.69	44.0	3800	178	+29
	29 After 16 days albumin intravenously	7.63	5.11	41.7	4288	219	+70
	37 After 8 days protein hydrolysate intravenously	6.68	4.34	42.0	4082	177	+28
	46 After 9 days normal diet	6.81	4.22	43.8	3793†	160	+11
	53 After 16 days normal diet	6.83	3.96	46.3	3876	153	+ 4
W. C.	1 Control	5.57	4.00	43.0	2651†	106	
	5 After 4 days protein hydrolysate intravenously	6.29	4.40	43.2	2630†	116	0
	During 6 intravenous 7 albumin 8 9	6.26 5.86 6.81 7.14	4.96 5.13 5.19 6.12	42.8 44.4 38.8 37.7	3305†	202	+56
	12 After 3 days oral albumin	6.79	5.42	39.8	3025†	164	+45
	19 After 7 days normal diet	7.29	5.27	43.8	2566†	135	+19
	33 After 21 days normal diet	6.39	4.27	42.4	2717†	116	= 0

* Albumin and globulin separations by electrophoresis (W. K. and D. R.) and by Hone precipitation (W. C.).
† Calculated from changes in hematocrit—red blood cell volume assumed constant.

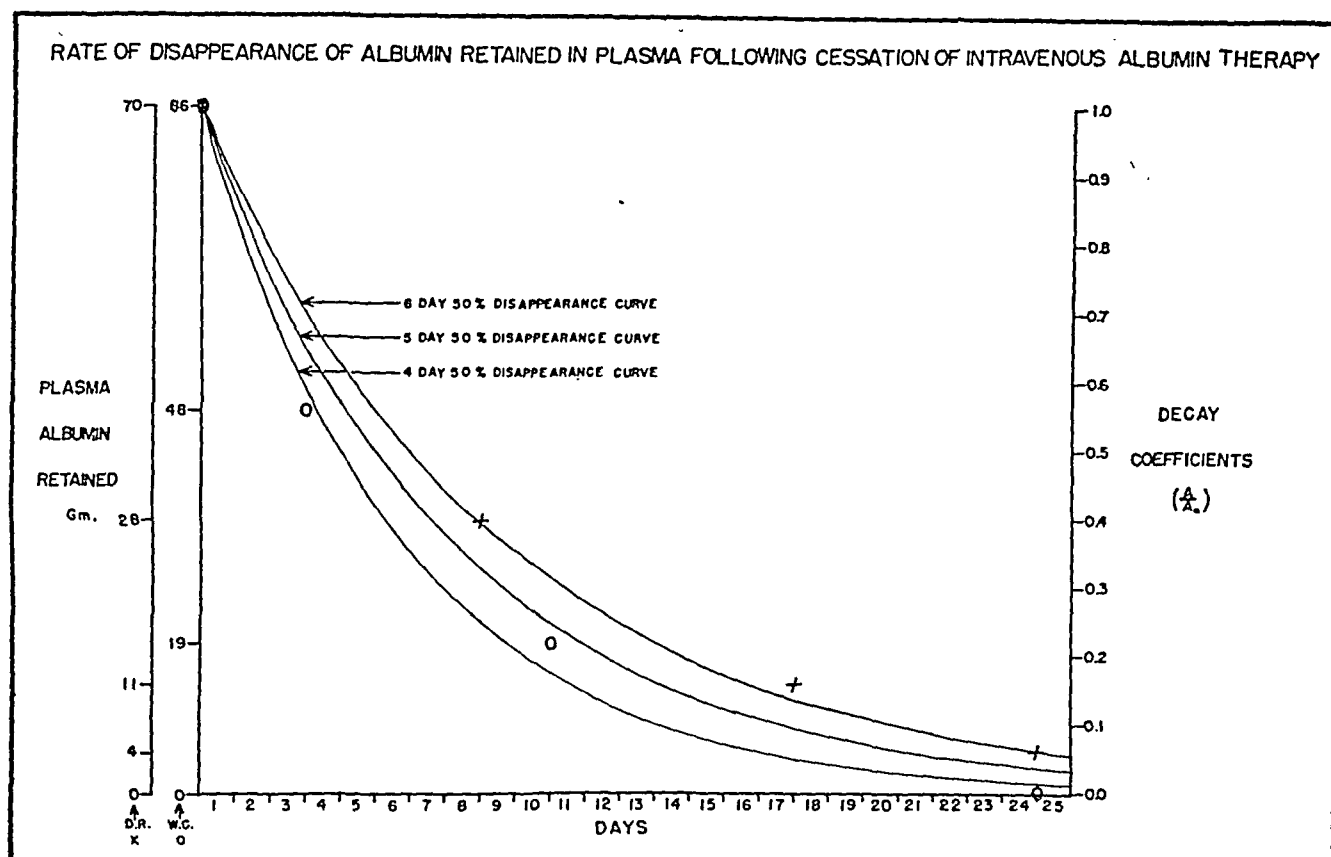


FIG. 6

so that by 24 days following the cessation of albumin administration the total circulating albumin had fallen to nearly the original value.

During the first period of casein hydrolysate administration, the subjects excreted somewhat more nitrogen (in terms of protein) than was administered (Table II, column 3). This is accounted for largely by the time required for approximate nitrogen equilibrium to be reached with the step-wise increase in hydrolysate administration (Figures 4 and 5). In contrast, during the period of albumin administration, both subjects retained at least 20 per cent of the protein administered

(Table II, column 4). Of the protein retained, 24 per cent and 46 per cent, respectively, was accounted for by increase in the plasma albumin (Table II, column 7). During the final period of casein hydrolysate administration, subject D. R. excreted slightly more nitrogen than was given.

Urinary tryptophane excretion was essentially normal (Figure 7a and 7b) until D. R. received the albumin supplemented with dl-tryptophane, 2 grams daily for 8 days. During these 8 days he excreted an excess of 5.16 grams. The quantity of casein hydrolysate used contains about 375 mgm. of l-tryptophane. It is apparent that only a small fraction of this tryptophane was excreted.

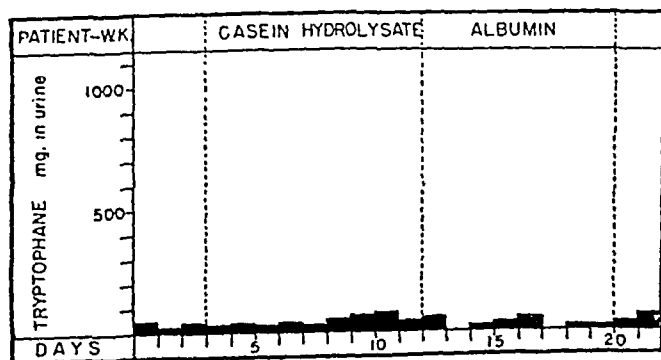


FIG. 7a

III. Protein administered both orally and intravenously (Figure 8, Table II)

In contrast to the period during which the 4 individuals received a small amount of protein (37.5 grams) daily, either orally or intravenously, and the one who received orally a moderate amount (50.0 grams) daily is another study during which the latter subject (W. C.) was given 75.0 grams daily. During the first period of 4 days when the protein was supplied intravenously as casein hydroly-

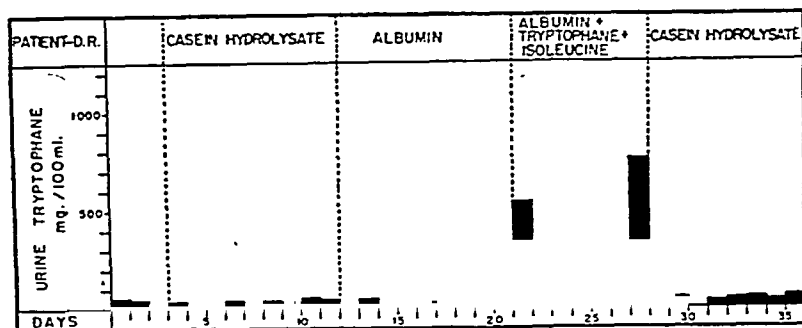


FIG. 7b

sate, the subject maintained a slight positive nitrogen balance amounting to 70 grams of protein for the period (Table II, column 3). There was a small rise in plasma albumin concentration. Of the nitrogen excreted daily during this period 0.28 gram was "free" alpha amino nitrogen, and 1.3 grams were "polypeptide nitrogen" determined as alpha amino nitrogen after hydrolysis of the urine. This amount of "polypeptide nitrogen" represents approximately 45 per cent of the polypeptide alpha amino nitrogen administered.

The day intravenous albumin was substituted for the casein hydrolysate, nitrogen excretion fell to approximately 3 grams per day, making the nitrogen balance about 8 grams positive per day, and totaling 205 grams of protein for the period (Table II, column 3). During this period there was a selective rise in the plasma albumin concentration of 1.72 grams per 100 cc. Plasma volume measurements were not satisfactory in this patient, but, assuming an unchanged red cell volume, it is possible to calculate the rise in plasma volume from the observed fall in hematocrit. Using these assumptions, roughly 40 per cent of the albumin retained was in the blood (Table II, column 7).

Nitrogen excretion during these 4 days represented 96 grams of protein. This would account for about 30 per cent of the albumin administered (301 grams) had it all come from this source, but if it is assumed that such a low nitrogen excretion is largely from "endogenous" protein catabolism, then up to 100 per cent of the albumin administered was retained (Table II). Thus, between 70 per cent and 100 per cent of the albumin administered was retained in the body, the greatest amount being extravascular and roughly 30 per cent in the blood plasma (Table II, column 6).

When an equivalent amount of albumin was given by mouth during the next 3 days, nitrogen excretion rose progressively so that on the third day approximate equilibrium was obtained. On the last day of the intravenous albumin administration and the first day that it was given orally there was slight proteinuria, 1.19 and 4.69 grams of protein, respectively.

The disappearance rate of the retained plasma albumin was somewhat more rapid in this subject than in subject D. R. The values for the total albumin increase (retention) in the plasma (Table

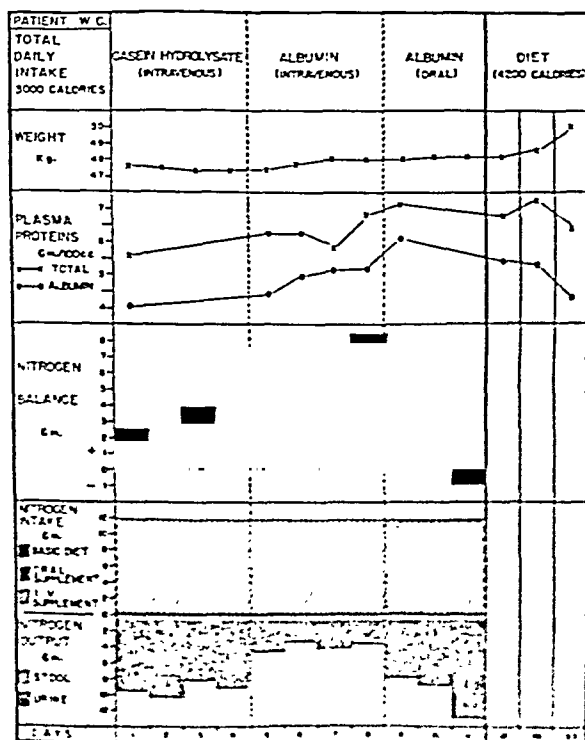


FIG. 8

III, column 6) determined at the conclusion of the intravenous albumin administration and the decrease at varying intervals thereafter roughly fall on a 4-day 50 per cent disappearance curve (Figure 6). The total circulating plasma albumin had returned to its original value approximately 3 weeks after the intravenous albumin therapy was discontinued.

DISCUSSION

A sufficient amount of a single complete protein as a sole source of food nitrogen in a normal healthy subject should maintain nitrogen equilibrium if the caloric intake is high enough to prevent utilization of protein for energy. The first 2 subjects reported here were in negative nitrogen balance throughout while receiving 37.5 grams of human albumin by mouth. The failure to maintain nitrogen equilibrium was apparently not due to the low content of tryptophane and isoleucine in human albumin (6, 7) since the addition of adequate amounts of these 2 amino acids did not change the nitrogen excretion. Because the third subject maintained a slight positive nitrogen balance on 50.0 grams of unsupplemented albumin by mouth (representing almost twice the quantity of protein per kilogram of body weight in view of this subject's small stature), it is suggested that the previous individuals received an insufficient quantity of protein. This quantitative deficiency is further borne out by the fact that neither subject reached true nitrogen equilibrium on the same amount of the presumably complete lactalbumin during the control period. The 37.5 grams of protein (albumin or lactalbumin) given these 2 subjects daily furnished slightly under 0.5 gram of protein per kilogram of body weight which borders on the minimum requirement (19). However, normal active individuals should theoretically maintain nitrogen equilibrium on less protein than this (20). The loss of weight in the 2 subjects not in nitrogen equilibrium was greater than could be accounted for by the nitrogen lost, and was probably due in part to mild dehydration associated with periods of diarrhea, particularly since both rapidly gained weight upon return to their normal dietary habits. It is possible that the caloric requirement in these subjects was greater than we supplied, even though they were given a minimum of 3200 calories daily—at least 40 calories per kilogram of body weight.

In contrast to the negative nitrogen balance when a critical level of albumin (37.5 grams) was given orally, a positive balance persisted during the intravenous administration of the same amount of albumin. Nitrogen excretion was low and approached the "irreducible minimum," and there was a selective rise in the plasma albumin concentration. Supplementation with tryptophane and isoleucine did not alter the positive nitrogen balance even though approximately 3 grams of the natural isomer of tryptophane was retained during the period of its administration.

Whipple, *et al.* (13) and Howland and Hawkins (14) found a positive nitrogen balance and an absence of significant nitrogen loss in the urine when plasma was given to dogs intravenously, and an increased excretion of nitrogen when plasma was given orally. They present these results as evidence that the injected plasma is more completely utilized by the body. The authors concluded that injected plasma proteins could replace tissue proteins without being broken down to their constituent amino acids, and suggested a modification of the protein entering the cells by means of a cleavage into large aggregates and reassembly into the normal cell proteins. We suggest an alternative interpretation: Whole protein (albumin) given intravenously to normal individuals diffuses first into the lymphatics where an equilibrium is established. It is then only slowly metabolized, finally breaking down gradually, and probably completely, into its constituent amino acids; these are either deaminated, the nitrogen released then being excreted, or are resynthesized into new tissue protein. The evidence and reasons for this interpretation are presented below.

Parenterally injected albumin and plasma proteins disappear rapidly from the blood stream (1, 21), but repeated injections are followed by an appreciable and readily detectable increase in the total circulating plasma proteins, as reported here and by others (5, 10, 12). It is clear that roughly 24 to 46 per cent of the protein retained in our subjects could be accounted for by the albumin increase in the blood plasma (Table II, column 7). It is likewise apparent that most of the other 54 to 76 per cent was not metabolized promptly and excreted in the urine, as was the case with protein entering the body in a hydrolyzed form (oral albumin and lactalbumin, and intravenous casein

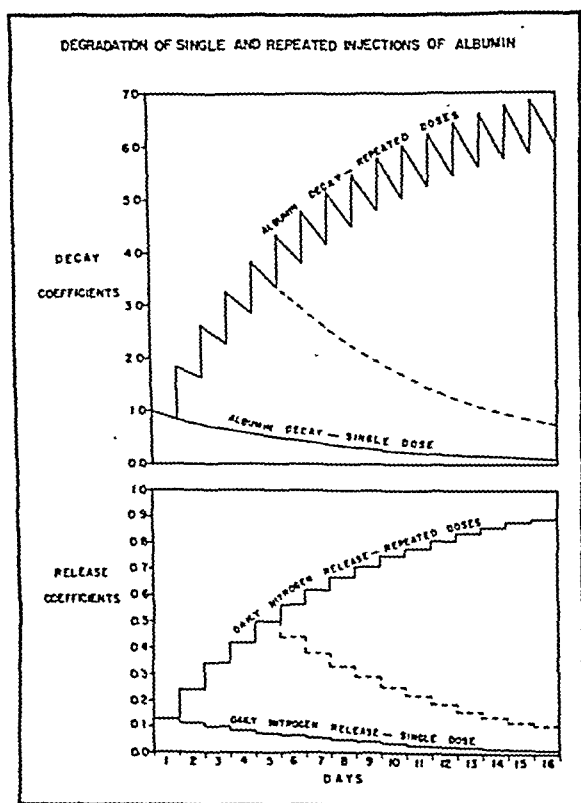


FIG. 9

hydrolysate). Presumably the albumin which left the circulating blood diffused through the capillaries into the interstitial spaces and entered the lymphatic system. The extensive studies of C. K. Drinker demonstrate that serum albumin and serum globulin "are normal constituents of lymph wherever it is collected, and there can be no doubt that they filter from the blood capillaries all over the body" (22). Labeled plasma proteins (23), foreign proteins (24), and dyes that adhere to proteins (23, 24) rapidly appear in the thoracic duct lymph after their intravenous injection. Also, the concentration of protein in liver lymph is practically identical to that of plasma (25), and we have observed a patient with a chylothorax in whom the lymph and plasma albumin concentrations were nearly identical (3.0 grams and 3.2 grams per 100 cc., respectively). As compared to hepatic and thoracic lymph, the protein content of edema fluid, transudates, and the peripheral lymph is lower, and the diffusion into these fluids of labeled plasma proteins (23) and dyes that adhere to proteins (23) occurs more slowly. Nevertheless, the larger volume of the interstitial and

lymphatic fluid as compared to blood plasma could account for most, if not all, of the albumin retained extravascularly in our subjects.

Following injections of radioactive labeled plasma proteins and radioactive dyes that adhere to proteins, Cope and Moore (23) demonstrated that an equilibrium (ratio of radioactivity to protein concentration) was attained between serum and thoracic lymph in about 6 hours, and peripheral (leg) lymph in 17 to 23 hours. Albumin presumably behaves in a similar manner, so that as the albumin concentration increases in the plasma during the period of intravenous albumin administration, that in the lymphatic system likewise rises.

After the albumin injections were discontinued in our subjects, the albumin retained in the plasma disappeared relatively slowly at a "50 per cent disappearance time" of roughly 4 to 6 days (Figure 6), so that return to the control plasma albumin content was not reached until approximately 3 weeks after resuming a normal diet. Others have observed that injected plasma protein and injected antibody protein disappeared from the plasma at a similar rate. Thus Fink *et al.* (26) injected plasma protein labeled with radioactive lysine into normal dogs. After an initially rapid disappearance from the plasma during the first day, approximately 50 per cent of the remaining labeled plasma protein disappeared in the next 5 days. Likewise, Heidelberger *et al.* (27) injected antibody protein into rabbits. Of the protein present in the plasma on day 2, 37 and 67 per cent (average 52 per cent) had disappeared by day 7. Although neither the disappearance from the plasma of labeled plasma protein injected into dogs (26) nor of antibody protein injected into rabbits (27) appeared to be logarithmic, in contrast to the apparent logarithmic disappearance of the retained plasma albumin in our subjects (Figure 6), the similarity is apparent in the rate of disappearance of albumin, plasma protein, and antibody protein from the plasma after intravenous injection. The rate of disappearance of albumin retained in the plasma of our subjects should be a measurable index of the disappearance of the albumin retained in the lymphatic system as well, for their fall will coincide since they are in equilibrium. Thus, the selective rise in the plasma albumin concentration which only slowly returned to the control value, as well as the positive nitrogen balance and mini-

mum nitrogen excretion in the well-nourished individuals reported here, has led us to believe that albumin administered intravenously is not promptly metabolized but degraded slowly.

To more clearly visualize the process of degradation as we understand it, we have constructed the diagram shown in Figure 9 which was calculated from the general equation of decay.¹⁰ The diagram shows the quantity of albumin that would remain "unchanged" in the body if albumin were given parenterally on 1 or on successive days, as well as the quantity of nitrogen that would be "released"

¹⁰ The authors gratefully acknowledge the assistance of Herbert Jaffe, Ph.D., in the following mathematical interpretation:

(a) *Albumin decay—single dose*: $A = A_0 e^{-kt}$, where A = quantity of albumin in body at any time, t , after initial dose; A_0 = quantity (dose) of albumin given initially; t = time in days after initial dose; and $k = 0.1386$ (50 per cent decay in 5 days). Values plotted in Figure 9 as decay coefficients $\left(\frac{A}{A_0}\right)$ where $\frac{A}{A_0} = e^{-kt}$. Coefficient at any time, t , following initial dose \times dose albumin given = quantity of albumin in body at time, t .

(b) *Daily nitrogen release—single dose*: Obtained by subtracting from the quantity of albumin present in body on any day the smaller quantity remaining 1 day later. Plotted as release coefficients, so that the coefficient at any time, t , following initial dose \times dose albumin nitrogen given = quantity nitrogen released from decay of albumin for that day.

(c) *Albumin decay—repeated doses*: By an expansion of the general equation of decay (Equation a), one obtains:

$$A_n = A_0(e^{-kt'}) + A_0(e^{-kt'})^2 + A_0(e^{-kt'})^3 + \dots \\ + A_0(e^{-kt'})^{n-1} + A_0(e^{-kt'})^n,$$

where A_n = quantity of albumin present in body after n , number doses; A_0 = quantity (dose) of albumin given initially and repeatedly on multiple dosage schedule; n = number of doses given; t' = interval between doses (one day for this experiment). Values plotted as decay coefficients $\left(\frac{A_n}{A_0}\right)$ where

$$\frac{A_n}{A_0} = (e^{-kt'}) + (e^{-kt'})^2 + (e^{-kt'})^3 + \dots \\ + (e^{-kt'})^{n-1} + (e^{-kt'})^n.$$

By multiplying the coefficient for any day by the dose of albumin given initially, one obtains the quantity of albumin present in the body on that day.

(d) *Daily nitrogen release—repeated doses*: Obtained by subtracting from the quantity of albumin present in the body after a dose is given the quantity present prior to giving the next dose (e.g., the amount decayed on that day). Coefficient for any day following initial dose of albumin \times dose albumin nitrogen given initially = quantity of nitrogen released from the decay of the albumin present in the body on that day.

each day from the degradation of the albumin still remaining in the body. The conditions for the data in Figure 9 are these: (1) The quantity of albumin given once or repeatedly is of equal dosage with equal intervals between doses; and (2) the albumin is assumed to decay at a 50 per cent disappearance time of 5 days (average of values in our subjects, Figure 6). For simplicity of presentation, it is assumed to break down at a constant and uniform rate, even though factors such as the nutritional state of the tissues, the plasma albumin level, the quantity of material injected, and alterations that may occur in the nutritional and physical properties of the albumin during processing might modify both the rapidity of diffusion from the blood stream into the extra-vascular compartments, and the degree and rate of degradation of the protein. Following the single injection of any given quantity of albumin there would be a steadily decreasing amount of albumin remaining "unchanged" in the body on successive days, so that on day 5 only one-half (50 per cent) of the original would remain. The quantity of nitrogen "released" from this breakdown of albumin would similarly decrease in amount on each subsequent day. When, however, repeated daily injections of the same dosage of albumin are given for several days, the quantity of albumin remaining "unchanged" in the body would increase in step-like fashion as would the amount of nitrogen "released." Both curves tend to become asymptotic after approximately 2 weeks of daily albumin injections so that the quantity of nitrogen released each day approaches the quantity of albumin nitrogen given daily. The dotted lines in Figure 9 show the decreasing quantities of albumin that would remain in the body and the diminishing amounts of nitrogen that would be released from its decay if albumin injections were discontinued after 5 days.

The question may now be asked: Is the intravenously administered albumin, which we believe is degraded slowly, broken down completely to its constituent amino acids? If it were, the amino acids released from the breakdown of the albumin would be available for deamination and excretion or resynthesis into tissue proteins, the amounts thus "converted" or "burned" depending upon the quantity of amino acids available and the needs of the body for protein. By employing the mathe-

mathematical data in Figure 9, it is possible to determine the quantity of nitrogen theoretically "released" in our 2 subjects who received daily the small amount of albumin (6.0 grams nitrogen) intravenously. Under these conditions one would expect to find 0.8 gram of nitrogen released on day 1 (0.13×6); 3.0 grams on day 5 (0.5×6); and 4.5 grams on day 10 (0.75×6). Therefore, when one administers on successive days only small amounts of albumin as a sole source of nitrogen (as in our subjects given 37.5 grams daily), the nitrogen released daily, even after several days, would no more than maintain the body protein requirements even if all of the released amino acids were converted into tissue protein. Thus one would not expect to detect a gradually increasing urinary nitrogen excretion—and, indeed, none was observed in our subjects during the 8 and 16 days of daily intravenous albumin therapy. Nor has Whipple *et al.* (13) noted a significantly increased nitrogen excretion in dogs receiving only moderate amounts of plasma intravenously as the sole source of nitrogen. However, had Whipple's basal diets contained adequate protein to fulfill the minimum nitrogen requirements, or had large quantities of plasma been given by vein, one might well have observed an increased nitrogen excretion. Indeed, when a constant oral diet containing at least 50 grams of protein was given by Albright (11) to his patients, an increased urinary excretion of nitrogen was observed by about the third day after plasma injections, and was not complete in the subsequent 1 to 3 weeks. Under these conditions, the oral nitrogen could be considered to fulfill the body protein requirements, the nitrogen "released" from the decay of the injected plasma protein would be "extra," and that quantity of nitrogen would appear in the urine. Furthermore, when large quantities of plasma protein were administered intravenously by Meyer *et al.* (12) to patients, and by Elman and Davey (10) to dogs, both groups noted a subsequent rise in the urinary nitrogen excretion resulting in an "apparent" negative nitrogen balance. The results of the latter investigators however, have been questioned since citrate was used as the anti-coagulant (28). The parenteral administration of large amounts of plasma as the sole source of nitrogen is analogous to small or moderate amounts of plasma plus a quantity of dietary protein which in itself could

fulfill the body protein needs. The most plausible explanation for the observed increase in urine nitrogen (shown by Meyer [12] to be non-protein-non-peptide nitrogen by nitrogen partition) that follows intravenous plasma administration is that the retained plasma protein is subsequently broken down to amino acids which, if present in excess of the body protein requirements, are then deaminated and the released nitrogen excreted. The critical observations of Albright (11) that "conversion" and "burning" of injected plasma protein do not start to an appreciable degree until several days after the plasma protein injections is readily explained by this hypothesis, for several days must elapse before the degradation of the injected plasma has released sufficient amino acids for resynthesis into tissue protein, and for those released in excess of these requirements to be deaminated and excreted in the urine.

Further evidence that the body less readily uses conjugated than "free" amino acids is found in the work of Christensen, Lynch, and Powers (29). These authors recently observed that in 3 to 6 hours following the intravenous administration of a partial protein hydrolysate ("Amigen") the plasma elevation of conjugated amino acids (polypeptides) was larger and more prolonged than that of free amino acids, despite the fact that two-thirds of the amino acids in this hydrolysate appeared to be free. Concomitantly only 2.4 to 6 per cent of the "free" alpha amino nitrogen administered appeared in the urine as contrasted to 36 to 53 per cent of that administered as "bound diffusible" (polypeptide) nitrogen. Our figures (in subject W. C.) are in agreement with these and suggest that most of the 1.3 grams of polypeptide amino nitrogen excreted came from the protein hydrolysate administered. These observations indicate that the polypeptides present in partially hydrolyzed protein given intravenously resemble albumin in that they are not promptly metabolized. In contrast to albumin, however, and probably due to their low molecular weight they are largely excreted in the urine rather than being primarily retained in the body. Considering the small loss of "free" alpha amino nitrogen following the intravenous administration of this hydrolysate, one might expect a completely hydrolyzed protein to be of greater nutritional value than a partially hydrolyzed protein.

Since parenterally administered albumin and plasma proteins are neither rapidly metabolized in the body nor excreted as such by the normal kidney, a positive nitrogen balance (as measured in terms of what goes in and what comes out) is practically inevitable, and can occur even in the "catabolic period" following injury.¹¹

Although not immediately available as a protein nutrient, adequate quantities of injected albumin and plasma can serve as a delayed nutrient, entering into the metabolic pool only slowly where they presumably fulfill all the body protein requirements. In fact, Whipple (30) has shown that dogs receiving plasma intravenously as the sole source of nitrogen have remained in perfect health for more than 3 months. Because of the slow and gradual release of amino acids from injected whole protein, more "efficient" utilization may occur, particularly when one considers this utilization extending over days instead of hours which is the case when the protein is orally ingested. Albright observed (11) that a portion of the retained plasma protein is eventually converted into tissue protein in the face of a constant oral diet which in itself contains adequate protein to maintain nitrogen equilibrium. This suggests that whole protein parenterally injected may actually increase the amount of body tissue normally formed.

SUMMARY

Normal human serum albumin was administered orally or intravenously to 5 normal individuals as a sole source of nitrogen for periods of from 7 to 16 days. Nitrogen balance and plasma protein concentration were studied and the effects of the administered albumin compared in each instance with those obtained with intravenous casein hydrolysate, oral lactalbumin, or oral human albumin.

Albumin administered orally in small amounts (37.5 grams daily) to 2 individuals failed to main-

tain nitrogen equilibrium itself or when supplemented with tryptophane and isoleucine. The lactalbumin preparation in the same amount also failed to maintain nitrogen equilibrium. There was no significant change in concentration of plasma proteins in this study.

Albumin administered orally in a larger quantity (50.0 grams daily) to 1 individual maintained weight and nitrogen balance, even though the albumin was not supplemented with tryptophane or isoleucine. Albumin in adequate amounts, therefore, contains all the essential amino acids required by man for maintenance of nitrogen balance.

The intravenously administered partial protein hydrolysate maintained a slightly positive nitrogen balance for very short periods of time and was associated in 1 subject with the excretion in the urine of 46 per cent of the polypeptide administered.

Albumin administered intravenously in small amounts (37.5 grams daily) maintained a positive nitrogen balance for 8 and 16 days in 2 individuals, and was associated with a selective rise in plasma albumin concentration. There was no significant difference in nitrogen balance when the albumin was supplemented with tryptophane or isoleucine.

Albumin administered intravenously in large amounts (75 grams daily) to 1 individual for 4 days produced a very marked positive nitrogen balance with minimum nitrogen excretion. This was in contrast to the effect of intravenous casein hydrolysate in a previous period and of albumin administered orally in a subsequent period, during both of which the nitrogen excretion approached the intake. The plasma albumin concentration rose 1.72 grams to an abnormally high level (6.12 grams per 100 cc.) during the period of intravenous albumin administration.

After the intravenous injections of albumin were discontinued in 2 subjects, the albumin retained in the plasma disappeared at a 50 per cent disappearance time of 4 to 6 days, so that by 3 weeks the level had fallen to nearly the original value.

The following mechanism for the metabolism of intravenously administered albumin in normal man would appear to explain the observations presented here and those of other investigators: When albumin is administered intravenously it

¹¹ The "inevitable" positive nitrogen balance will occur only when the quantity of whole protein nitrogen injected exceeds the loss of nitrogen in the urine that would occur on a protein-free diet. Thus, one would anticipate a negative balance if but 10 grams of albumin were given to a subject whose endogenous urinary loss exceeded 1.6 grams of nitrogen daily, or if 100 grams of albumin were given to a patient whose catabolic urinary excretion was greater than 16 grams of nitrogen daily.

rapidly disappears from the plasma by diffusion into the lymphatic system, and an equilibrium or balance between the vascular and extra-vascular albumin is soon established. After repeated injections there is an increase in the total plasma albumin and the lymphatic albumin. After the albumin infusions are discontinued there is a disappearance of the albumin retained in the plasma which is an index of the rate of disappearance of the albumin retained in a state of balance in both the vascular and extra-vascular compartments. The albumin which is retained in the body disappears by a process of degradation or decay by which nitrogen is released as amino acids. The amino acids released from the slow breakdown of albumin are either resynthesized into tissue protein or deaminated and excreted in the urine as non-protein nitrogen, the amounts thus "converted" or "burned" depending upon the needs of the body for protein and the quantity of amino acids released. A positive nitrogen balance will occur when albumin is given intravenously since the albumin is neither excreted by the normal kidney nor rapidly metabolized in the body. Parenterally administered albumin can serve as a body protein nutrient, and perhaps a very good one, but its action is delayed, entering into the metabolic pool only slowly, as contrasted to protein which enters the body in a hydrolyzed form. It is because of the properties that tend for slowness of utilization that albumin is an ideal substance for temporarily increasing the plasma albumin concentration and the oncotic pressure of the blood.

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THE EFFECTS OF INTRAVENOUS INJECTION OF CONCENTRATED HUMAN SERUM ALBUMIN UPON BLOOD PLASMA, ASCITES AND RENAL FUNCTIONS IN THREE PATIENTS WITH CIRRHOSIS OF THE LIVER¹

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The mechanism of ascites formation has been a subject of interest for many years and remains a challenge to the investigator. In previous reports (1, 2) on 61 patients with cirrhosis of the liver, it was pointed out that those with ascites have lower values for serum albumin than do those without ascites and that the tendency to diuresis (and the loss of ascites) was correlated with a rise of the serum albumin level. The "critical" point of diuresis was found to be about 3.1 grams per cent. However, the slowness of diuresis, frequently taking place over a period of several months, did not provide a sharp endpoint for establishing a critical level. Although other factors, such as an increased portal capillary pressure, were considered to be contributory, it seemed likely that the colloid osmotic pressure of the plasma was the chief determinant for the presence or absence of ascites.

There are several objections to this concept: the rise of serum albumin did not regularly precede a diuresis although it appeared to accompany the phenomenon; wider experience in a series of 115 cases with ascites revealed 7 cases in which diuresis failed to take place even though the serum albumin rose above 3.5 grams per cent; and, conversely, there were 3 cases in which diuresis took place when values of serum albumin were less than 3.0 grams per cent. Ralli and her associates (3) have cited instances in which diuresis with loss of ascites took place at low values for serum albumin in patients receiving intravenous liver extract. Furthermore, they presented

evidence suggesting that anti-diuretic substances might play a role in the formation of ascites.

Concentrated human serum albumin solution has provided a means for testing directly the relation of serum albumin and colloid osmotic pressure to ascites formation, for by injecting albumin, the colloid osmotic pressure of the plasma can be raised abruptly. Concentrated serum albumin has been employed by others with the aim of determining its place as a therapeutic agent for cirrhosis of the liver with ascites. Janeway and his associates (4) reported that standard concentrated human albumin solution administered to 6 patients with portal cirrhosis produced no apparent change in ascites formation. The total dosage in 2 instances was 350 and 950 grams. Thorn and his co-workers (5) administered a salt-poor albumin solution to 5 patients with various types of liver cirrhosis associated with ascites and edema. A transient diuresis occurred in all 5 patients after a single infusion of 50 grams and after 3 daily infusions of 50 grams of albumin. The fluid seemed to have been mobilized from the peripheral edema rather than from the ascites. Two of these patients subsequently lost their ascites after receiving a total dosage of 450 and 500 grams in 27 and 10 days, respectively. Gibson (6) and Kunkel (7) also report that prompt diuresis and loss of ascites occurred in certain patients after the administration of standard concentrated human albumin solution.

The present study was designed to make direct measurement of several factors that might be altered by the injection of concentrated albumin solution. The plan of this experiment was inspired by a similar experiment performed by Luescher (8) upon patients with nephrosis. Attention was directed at 2 aspects of the problem: (1) measure-

¹ The work described in this paper was done under contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

ment of factors affecting osmotic relations between blood plasma and ascites, and (2) measurement of factors involved in renal function.

The present studies indicate that ascites formation is not determined solely by the level of the plasma colloid osmotic pressure, and they suggest that other factors, such as permeability of the portal vascular bed and alterations in water metabolism, are involved.

PATIENT MATERIAL AND PROCEDURE

The 3 patients selected for study had been in the hospital for 7, 8, and 21 months preceding the experiment and were stabilized with regard to their disease. That is, they manifested no tendency towards regression or progression of the disease at the time of these observations. All 3 had persistent ascites, hypoalbuminemia, and minimal edema. Patients D. and G. appeared to have classical Laennec's cirrhosis. Biopsies of their livers showed the characteristic picture of this disease. Patient N. appeared to have healed yellow atrophy (toxic or post-necrotic cirrhosis) as a sequel to infectious hepatitis. Laparotomy subsequent to these studies revealed a very small cirrhotic liver. However, no biopsy was performed. Summaries of their case histories are appended.

The patients were kept in a separate room and were fed a weighed diet containing approximately Protein 120, Carbohydrate 365, Fat 110 grams during a control period of 4 weeks and throughout the experimental period. The salt content of the diet was estimated to be about 5 grams daily. Fluid intake was not restricted. No medications were given during the experimental period.

Measurements were made of transient effects following a single intravenous injection as well as the cumulative effects of 13 to 16 daily injections of concentrated human serum albumin solution. At the time of the first and the tenth injection, studies of renal function were carried out in addition to various determinations on the blood and ascitic fluid. Fasting blood specimens were obtained each day preceding the infusion of albumin solution. The daily body weight, fluid intake and output were recorded.

The albumin solution was administered by gravity with the standard infusion set used by the Medical Corps of the U. S. Navy. A daily infusion of 200 cc. of 25 per cent concentrated albumin solution in 0.3 M. NaCl² was administered in from 30 to 45 minutes. No untoward reactions occurred. The daily infusions were administered for 13, 14, and 16 days, respectively, in the 3 patients.

METHODS

During the control period and at intervals during and after the infusions of albumin solution measurements were made as follows:

² The material was prepared at the Harvard Fractionation Laboratories, Dept. of Physical Chemistry, from blood collected by the American Red Cross.

Blood: Cell counts, hematocrit, erythrocyte sedimentation rate (9), plasma protein concentration and partition (10), plasma colloid osmotic pressure (11), plasma volume (12).

Ascitic fluid: Cell counts, protein concentration and partitions, fluid volume (13), colloid osmotic pressure.

Urine: Qualitative analysis, non-protein nitrogen, 24-hour albumin excretion, assays of anti-diuretic substance (14).

Renal function: Inulin and p-amino hippuric acid clearance, and chloride excretion. Stable plasma concentrations of inulin and p-amino hippuric acid were maintained by an infusion. Urine was collected through a many-eyed catheter. Urine collection periods were generally of 20 minutes and were terminated by a bladder wash. Inulin was measured by a modification of the Harrison method (15), and p-amino hippuric acid by the Bratton and Marshall reaction (16).

Liver function: Bromsulfalein dye test, cephalin flocculation reaction, serum bilirubin, urine urobilinogen excretion, serum cholesterol, and prothrombin time. The methods employed for testing liver functions have been described in a recent report (17).

Nitrogen content of the food was estimated from standard tables. Nitrogen determinations on blood and urine were done by the micro-Kjeldahl method. Aliquots of 3 daily

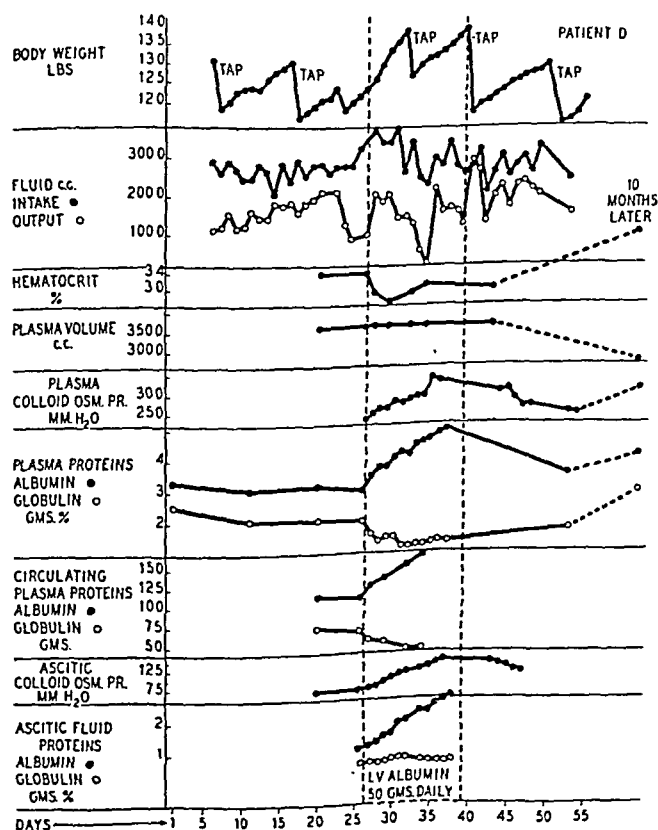


FIG. 1. EFFECTS OF MULTIPLE INJECTIONS OF CONCENTRATED HUMAN SERUM ALBUMIN IN PATIENT D

Note the changes observed 10 months later, at a time of spontaneous diuresis and disappearance of ascites.

urines were pooled and the values for nitrogen averaged. Fecal nitrogen was assumed to be constant.

RESULTS

After a single injection of concentrated human serum albumin in 3 patients with cirrhosis only moderate changes were observed. A slight rise occurred in the concentration of serum albumin, plasma colloid osmotic pressure, and plasma volume with an associated fall in the hematocrit. The protein constituents and colloid osmotic pressures of the ascitic fluid were unchanged during the first 24 hours after injection.

The effects of multiple injections of concentrated human serum albumin upon these measurements are illustrated by Figures 1, 2 and 3.

Fluid balance. In general the formation of ascites, as shown by weight gain and the frequency of abdominal taps, was not significantly altered. In patient D. (Figure 1) there was slightly increased accumulation of ascites during the experimental period. This pattern may have been com-

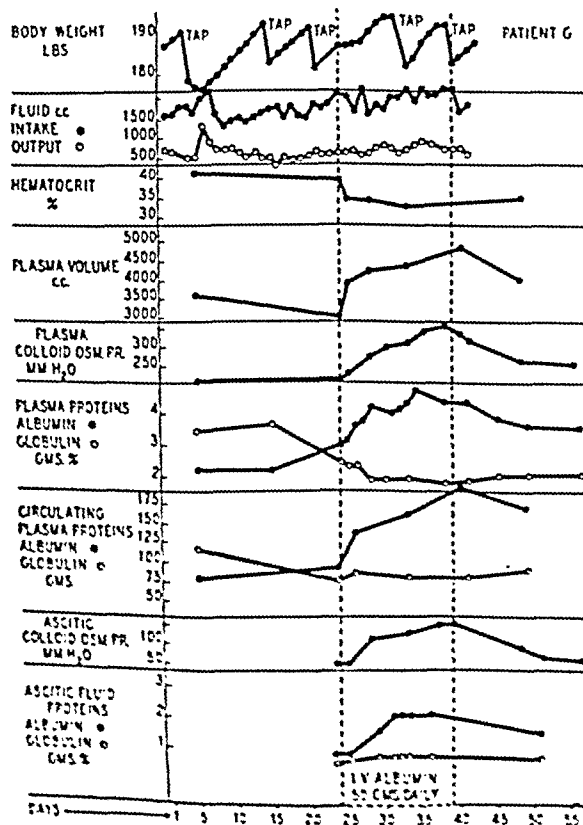


FIG. 2. EFFECTS OF MULTIPLE INJECTIONS OF CONCENTRATED HUMAN SERUM ALBUMIN IN PATIENT G

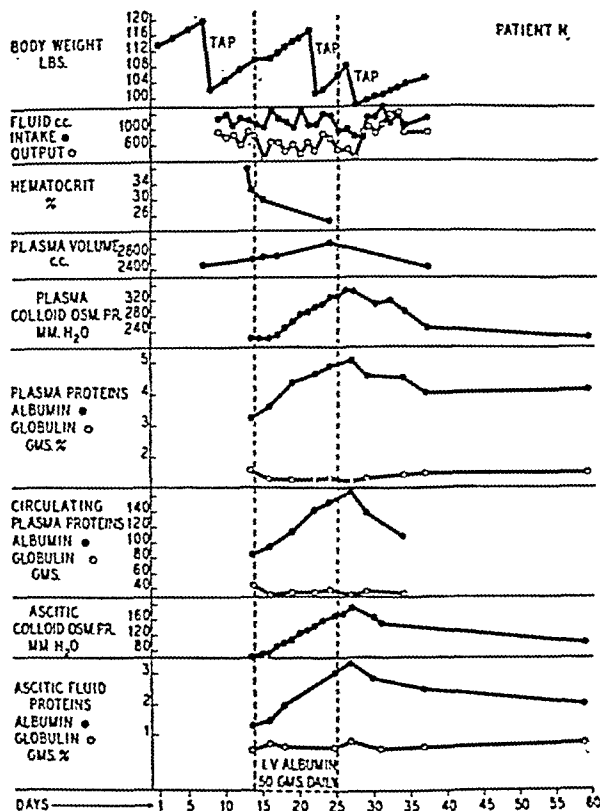


FIG. 3. EFFECTS OF MULTIPLE INJECTIONS OF CONCENTRATED HUMAN SERUM ALBUMIN IN PATIENT N

plicated by menstruation which took place towards the end of the experimental period. The relationship of urine output to fluid intake was unchanged by the injections of albumin in the 3 patients.

Plasma volume. The initial high values for plasma volume are in agreement with the findings of Perera (18). After injection of albumin solution there was a measurable increase in plasma volume in 2 instances. The lack of measurable change in patient D. is difficult to explain. The increased values probably are not attributable to loss of dye into the ascites, since there was a lag of 24 hours before it appeared in the ascitic fluid. None appeared in the urine. However, this does not exclude the possibility of the dye being localized in other areas. It is of interest that 10 months after completion of this study the plasma volume had declined from 3600 to 2700 cc. in patient D. shortly after there had been a spontaneous diuresis and loss of ascites.

Serum albumin and colloid osmotic pressure. In each case there was a progressive rise in the concentration of serum albumin and in total clon-

lating albumin (*i.e.* concentration \times plasma volume) during the administration of concentrated serum albumin, with a return to values only slightly higher than the initial levels in 2 to 3 weeks after cessation of therapy. The plasma colloid osmotic pressure showed a rise and fall parallel to that of the serum albumin. No diuresis took place even though normal values for serum albumin and colloid osmotic pressure were maintained for 10 days in each case. Moreover, in patient D., who failed to lose ascites when the serum albumin value of 4.2 grams per cent (colloid osmotic pressure 340 mm. H_2O) was attained, a "spontaneous" diuresis occurred 9 months later when the serum albumin level was 3.7 grams per cent (colloid osmotic pressure 290 mm. H_2O).

Serum globulin. Decrease in the concentration of serum globulin, observed in all 3 cases, was due apparently to hemodilution (increased plasma volume) since the total circulating globulin was relatively constant.³

³ The slight decrease of circulating globulin in patient D. may have been due to error in the estimation of plasma volume in this case.

Ascites. There was a rise both in the concentration of albumin and in the colloid osmotic pressure of ascitic fluid which paralleled the changes occurring in the plasma, but with a lag of about 24 hours. This is shown in Figures 1, 2, and 3. An increase in the ascitic fluid albumin, after the intravenous injection of concentrated albumin solution, was observed in earlier studies by Thorn and his colleagues (5). This finding may explain in part the failure of albumin injections to modify ascites formation in these patients. Because of the increased leakage of protein into the ascitic fluid the gradient between the colloid osmotic pressure of plasma and that of ascitic fluid was only slightly increased. Conditions therefore did not favor the resorption of fluid from the abdominal cavity. A detailed study of factors involved in the transfer of fluid between the plasma and ascitic fluid is presented in a separate report (13).

Renal functions. Values obtained for glomerular filtration rate and plasma flow, measured respectively by the inulin and para-amino hippuric acid clearances, are shown in Table I. After the first injection of concentrated albumin there was a

TABLE I
*Renal functions after single and multiple intravenous injections of concentrated albumin solution**

Patient	Date	Experimental period	Collection periods (No.)	Urine flow	Inulin clearance	P.A.H. clearance	Filtrate fraction	Plasma chloride	Chloride excretion
				cc. per min.	cc. per min.	cc. per min.	per cent	m. eq. per liter	m. eq. per min. $\times 10^{-3}$
D. Surface area 1.48	7/3/45	Control	2	1.16	122	512	23.7		
		1 hr. p. alb. no. 1	2	3.58	163	842	19.4		
	7/12/45	24 hr. p. alb. no. 9	2	1.55	190	934	20.3	115	16
	4/8/46	9 mo. later (spontaneous diuresis)	3	1.55	134	1233	10.8	111	259†
G. Surface area 1.93	4/26/45	Control	2	0.68	190	752	25.2	108	4
	5/14/45	Control	2	0.43	194	821	23.6	113	14
		1 hr. p. alb. no. 1	2	0.59	198	1250	15.9	114	36
		2 hr. p. alb. no. 1	1	0.95	227	1900	12.0	112	77
	5/23/45	24 hr. p. alb. no. 9	2	0.49	213	870	24.		
		2 hr. p. alb. no. 10	2	0.97	296	1404	21.		
		3 hr. p. alb. no. 10	1	0.62	203	985	20.6		
	10/18/46	5 mo. later (ascites present)	2	0.84	216	658	32.		
N. Surface area 1.42	5/14/46	Control	3	2.1	75	356	21.	111	41
		1 hr. p. alb. no. 1	2	5.95	85	503	16.9	113	308†
		3 hr. p. alb. no. 1	2	1.15	112	607	18.4	112	135
	5/24/46	24 hr. p. alb. no. 9	2	0.59	113	528	21.	109	25
		1 hr. p. alb. no. 10	2	1.87	104	592	17.5	111	80
		3 hr. p. alb. no. 10	2	0.51	120	645	18.6	110	23

* The initial glomerular filtration rates of patients D., G., and N. when corrected to the surface area of 1.73 sq. m. were 142, 172, and 91; the renal plasma flows were 600, 709, and 434. These values were within the normal range.

† Note increased chloride excretion during spontaneous diuresis in patient D. and during transient diuresis after albumin injection in patient N.

TABLE II

Assay for anti-diuretic substances in urine of a normal subject and patients with cirrhosis

Date	Normal subject excretion of		Saline control excretion of		Patient G. excretion of		Patient D. excretion of		Patient N. excretion of	
	25 per cent	50 per cent	25 per cent	50 per cent	25 per cent	50 per cent	25 per cent	50 per cent	25 per cent	50 per cent
	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
4-8-45	78 66	100 94			114 130	174 >180	98 108	>180 180		
4-21-45			76 102	140 136	88 104	130 166				
5-15-45	122 82	152 128	62 65	92 94	>180 114	>180 180				
5-22-45	108 84	170 132	83 75	113 104	92 126	122 156				
5-27-45	107 102	140 154	87 85	108 112	92 96	133 124				
6-3-45	84 86	118 102	78 78	102 102	94 96	128 132				
6-19-45			94 92	122 120	132 132	184 178				
6-26-45			94	146	160 110	>180 156	146 170	180 180		
7-1-45			82	106			120 152	154 180		
7-12-45			36 36	58 74			138 136	180 162		
7-16-45			same control				112 122	146 150		
4-17-46			68 90	96 116			86 88	110 116		
4-23-46			76 76	96 102	72 76	102 96	80 76	104 112		
5-12-46	65 75	90 100							94 92	130 120
5-24-46	76 82	100 108							146 138	>180 >180
5-26-46	same control								112 128	144 165
6-24-46	80 80	125 128							142 >180	180 >180

The table shows the time required for rats to excrete 25 and 50 per cent of the hydrating dose after intraperitoneal injection with urinary concentrates. Two groups of 3 male rats were employed for each specimen. Patient G. received 800 grams of concentrated albumin solution between 5-14-45 and 5-29-45. Ascites was present throughout the whole period of observation. Patient D. received 700 grams of concentrated albumin solution between 7-3-45 and 7-16-45. In April 1946 ascites could no longer be detected. Patient N. received 650 grams of concentrated albumin solution between 5-14-46 and 5-26-46. Ascites was present throughout the whole period of observation.

consistent rise in both of these functions. Values for glomerular filtration rate and plasma flow preceding the tenth injection also were higher than the control values preceding albumin therapy, thus

indicating a cumulative effect from the preceding 9 injections. Since the filtrate fraction (i.e. ratio of glomerular filtration rate to plasma flow) tended to decrease after the injections of albumin, it ap-

pears that there was a greater increase of plasma flow than of glomerular filtration.

A transitory diuresis of about 1 hour's duration was observed in patients N. and D. after the first injection of concentrated albumin solution. The diuresis was accompanied by an increased rate of chloride excretion in patient N. No measurement of chloride excretion was made in patient D. at that time. However, the latter patient showed a sharp increase in this function 9 months later, when she was having a spontaneous diuresis.

Anti-diuretic substance. Urines collected preceding, during, and after the injection of concentrated albumin were assayed for anti-diuretic substances according to the method of Burn as modified by Ham and Landis (14). Each specimen was tested on 2 groups of 3 rats and compared to a control group injected with physiological saline. The values obtained in patients D. and G. are shown in Table II. Each column represents the time required for hydrated rats to excrete 25 and 50 per cent of water administered by gavage, directly after intraperitoneal injection with the urine concentrate or with saline. Normally 50 per cent of the "hydrating" dose is excreted within 90 to 110 minutes.

Although patients D. and G. both had Laennec's cirrhosis and ascites, the assay of 1 patient's urine showed no marked deviation from normal values whereas the other (D.) showed a constantly delayed response. At the time of a spontaneous diuresis in the latter patient, occurring 9 months after albumin therapy, the urine assay showed normal values. The change from a delayed to a normal response in this patient was consistent with the findings of Ralli and her associates (3). The urine concentrate of patient N. (toxic cirrhosis) showed some anti-diuretic activity. The injections of albumin produced no significant alteration in the excretion of anti-diuretic substance in the 3 patients.

In 2 other patients with Laennec's cirrhosis and longstanding ascites, repeated assays of the urine showed varying results, from minimal to marked anti-diuretic activity. In 3 patients with diseases unattended by water retention, delayed responses were at times observed. This variability made interpretation difficult. The presence of hot weather (as pointed out by others) may have exerted an effect in certain instances. For example, the most

delayed responses of patient D. took place in June and July.

Because of the lack of homogeneity of the urine concentrate and the technical difficulties involved, the results of this test have been uncertain in our hands.

Nitrogen metabolism. The loss of albumin in the urine was negligible. The average daily urinary excretion of albumin for the week preceding therapy was 0.12, 0.19, and 0.15 grams for the 3 patients. During the 2-week period of injections the average daily excretion was 0.15, 0.37, and 0.48 grams, respectively.

However, there was considerable loss of albumin into the ascitic fluid. This was estimated by subtracting the total ascitic fluid albumin (volume \times concentration) at the beginning of the period from the total ascitic albumin at the end of the period. Preceding albumin therapy the average daily loss of albumin into the ascitic fluid was 9, 8, and 8 grams, respectively, for the 3 patients. During the period of albumin therapy the corresponding average daily losses were 25, 32, and 21 grams. The increased daily loss of albumin into the ascitic fluid during therapy represents 25 to 50 per cent of the albumin injected.

Nitrogen balance was determined in 2 patients. Nitrogen lost as albumin into the ascitic fluid was subtracted from the intake value. Prior to the administration of albumin solution, the daily intake of nitrogen exceeded the output by 2.6 and 5.2 grams. During the period of albumin therapy the daily intake exceeded the output by 7.0 and 9.9 grams, respectively. This increased retention of nitrogen during albumin therapy has been noted by Janeway, Thorn and their associates (4, 5). The explanation is not clear. There would appear to be 2 possibilities: either the material was diverted as such to extra-cellular fluid compartments or it was utilized to replace depleted cellular proteins. The fact that these patients were in positive nitrogen balance prior to albumin therapy suggests that there may have been depletion of tissue proteins.

Liver function. Several tests of liver function were performed preceding, during, and after the period of administration of concentrated albumin solution. No significant changes were noted in the bromsulfalein dye test, urine urobilinogen ex-

cretion, serum cholesterol, cephalin flocculation test, or prothrombin times.

DISCUSSION

There would appear to be at least 2 aspects to the mechanism of ascites formation in cirrhosis of the liver; namely, (1) factors influencing the localization of fluid within the abdominal cavity and (2) systemic factors favoring the retention of fluid in liver disease in general.

In a previous paper on this subject (1), it was suggested that the formation of ascites was dependent on the combined effect of lowered plasma osmotic pressure and increased portal venous pressure. Even though a fair correlation exists in the large majority of cases between the level of serum albumin and the presence or absence of ascites, this correlation does not necessarily imply a causal relationship. Indeed the present studies reveal that ascites formation is not determined by the level of the plasma colloid osmotic pressure alone and that other factors, such as the permeability of the portal vascular bed, may be important. However, it does not follow that the plasma colloid osmotic pressure has no bearing upon the formation of ascites. Rather, it may be considered as one of several factors, as demonstrated in a separate report (13).

In addition to the localization of fluid within the abdominal cavity, there is a peculiar capacity of the patient with cirrhosis of the liver and with other types of liver disease to retain water (19, 20). This phenomenon could be due to some factor operating within the extra-renal tissues or to an abnormal resorption of salt and water by the kidneys.

Reports by Ralli and her colleagues (3) have suggested that hormonal control of renal function may be the key to the problem, since the urine of patients with liver cirrhosis and ascites contained an anti-diuretic substance when tested in rats. However, it is not implicit that an anti-diuretic substance obtained from the urine is the same substance that is responsible for retention of fluid by the patient. In our experience a correlation between the presence of ascites and the presence of this anti-diuretic substance does not always exist. Further work in this field should help clarify this problem.

The retention of salt and water by these patients is not the result of impaired glomerular filtration, since these values were within normal limits in the 3 patients. The renal tubules apparently were able to reabsorb the greater amount of salt delivered to them by the heightened rate of filtration after the injections of albumin. The transient chloruresis in patient N., occurring when the filtration rate was increased, suggests that it is possible at times to exceed the reabsorptive capacity of the tubules for chloride.

The present studies do not establish the critical factors necessary to initiate sustained diuresis. It is quite possible that more sustained diuresis could have been achieved by the administration of larger amounts of concentrated albumin solution. The refractoriness to therapy of these 3 patients may have been related to the severity and chronicity of the disease process.

SUMMARY AND CONCLUSIONS

1. Single and multiple intravenous injections of concentrated human serum albumin in 3 patients with cirrhosis of the liver, hypoalbuminemia and longstanding ascites produced neither sustained diuresis nor disappearance of ascites. The clinical status of the patients and tests of liver function showed no apparent changes.

2. After single and multiple injections of the albumin solution the following effects were observed:

- (a) increase in circulating plasma albumin without apparent change in circulating globulin;
- (b) increase of colloid osmotic pressure of plasma to normal values;
- (c) concomitant increase in albumin concentration and colloid osmotic pressure of the ascitic fluid;
- (d) increase in plasma volume;
- (e) increase in glomerular filtration rate and in renal plasma flow;
- (f) increase in urinary chloride excretion at the time of transient diuresis.

3. The rate of transfer of albumin from plasma into the ascitic fluid increased 3-fold during the period of albumin therapy.

4. The intravenous injection of concentrated human serum albumin produced no significant

changes in the excretion of anti-diuretic substances.

5. The present studies indicate that ascites formation is not determined solely by the level of the plasma colloid osmotic pressure. They suggest the participation of other factors, such as changes in the permeability of the portal vascular bed and in salt and water metabolism.

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CASE HISTORIES

Patient D.

A 36 year old, Irish housewife with a history of alcoholism and grossly inadequate diet entered the Presbyterian Hospital 11/7/44 because of abdominal swelling. Peripheral neuritis had complicated a full term delivery 9 years previously. For several years there had been anorexia, weight loss, abdominal pain, paresthesias of feet and amenorrhea. For about 1 month fever, abdominal swelling, clay-colored stools, dark urine were observed. After a stay of 1 week at the Presbyterian Hospital she was transferred for study.

Examination disclosed a disoriented, irrational, emaciated woman, with marked icterus and sickly odor on breath. She exhibited an acneic rash on the face, prominent vascular spiders on face and neck, signs of cheilitis, glossitis, dilated superficial abdominal and thoracic veins, palpable liver and spleen, massive ascites, peripheral edema, and signs of polyneuritis. In addition there was an abscess of the right thigh.

Temperature 100° F.; pulse rate 90 per min.; respirations 22 per min.; blood pressure 104/72 mm. Hg; the initial laboratory data were as follows: Rbc 3.0 million per c. mm.; Hb. 10.8 grams per cent; Wbc 21,000 per c. mm. with 82 per cent polys (stabs 24); serum bilirubin 3.4 mgm. per cent; blood sugar 91 mgm. per cent; non-protein nitrogen 28 mgm. per cent; cephalin flocculation 3+; bromsulfalein 50 per cent in 30 min.; prothrombin 19 sec.; serum cholesterol 118 mgm. per cent; serum albumin 2.3 grams per cent; serum globulin 4.6 grams per cent; Wassermann negative. Urine negative except for pyuria.

For 2 weeks the patient was fed by tube with eggnogs (containing yeast) and fruit juices (calories 3000); and she was injected daily with thiamin (100 mgm.), nicotinamide (300 mgm.) and riboflavin (50 mgm.). Because of the fever and persistent leucocytosis she also received sulfadiazine therapy. On 11/30/44 the abscess of her thigh was incised and drained (*B. coli*). The patient showed marked general improvement, shown by subsidence of fever and jaundice, decreased number of spiders, gain in weight, strength and return of menses. However ascites formation required tapping at 10 to 14 day intervals. Puncture biopsy of the liver on 3/1/45 showed histologic changes of Laennec's cirrhosis.

Eight months after entry, trial of intravenous albumin therapy was made. At this time (7/3/45) the laboratory studies showed Rbc 3.3 million per c. mm., Hb. 10.7 grams per cent, Wbc 6400 c. mm., serum albumin 3.2 gram per cent, serum globulin 2.0 grams per cent, non-protein nitrogen 22 mgm. per cent, serum bilirubin 0.7 mgm. per cent. Flocculation test negative; prothrombin 20 sec.; urine urobilinogen 1.5 Ehrlich units per hour; serum cholesterol 175 mgm. per cent; bromsulfalein 54 per cent in 30 min. Alkaline phosphatase 11.8 Bodansky units; erythrocyte sedimentation rate 38 mm. per hour.

With albumin therapy the serum albumin level rose from 3.2 to a peak of 5.0 grams per cent on 7/14/45 and subsiding after 2 weeks to 3.5 grams per cent. Blood counts and tests of liver function performed at this time and during the next 3 months showed no significant changes from those preceding albumin therapy. Ascites formation continued.

In the ensuing 9 months when the patient received a highly nutritious diet and intravenous liver extract, there was progressive clinical improvement. The liver edge receded; the spleen was no longer palpable; ascites gradually disappeared. She was discharged July 1946, 19 months after entry. At this time laboratory data showed: Rbc 4.1 million per c. mm.; Hb. 15 grams per cent; serum albumin 4.3 grams per cent; serum globulin 2.5 grams per cent; serum bilirubin 0.5 mgm. per cent; flocculation test negative; urine urobilinogen 0.56 Ehrlich units per hour; bromsulfalein test 33 per cent in 30 min.; cholesterol 160 mgm. per cent (ester 103). When seen in the Out-Patient Clinic in February 1947, she was ambulatory and well.

Patient N.

A 37 year old, American-born housewife entered St. Vincent's Hospital in February 1945 because of abdominal swelling. Six years previously, after her last pregnancy she took "reducing pills" and her weight fell from 155 to 103 lbs. in 2 months, leaving her in a debilitated state. On advice of her doctor she regained 20 lbs. and then felt much improved. During the past 4 years, while estranged from her husband, her appetite decreased markedly. Although the food intake was meager, her diet was fairly well balanced. There was no story of alcoholism. Her husband and 5 children are living and well.

Her present illness began abruptly in January 1945, with weakness, nausea, vomiting, abdominal pain, jaundice, clay-colored stools, and dark urine in association with a severe upper respiratory infection. Rapid swelling of the abdomen appeared 2 weeks later. The chief findings at St. Vincent's Hospital included jaundice, ascites, and an enlarged tender liver. The patient was given a high protein, high carbohydrate, low fat diet. Because of persistent ascites formation an anastomosis of the right saphenous vein to the peritoneum was performed but without benefit. Ascites accumulated rapidly, requiring paracentesis at intervals of 3 weeks. The patient was transferred to another hospital and thence to the Columbia

Research Service for further care on 10/16/45, which was 10 months after the onset of her illness.

Examination showed a thin, frail, middle aged woman whose abdomen was greatly distended with fluid. Temperature 99° F.; pulse 80 per min.; blood pressure 120/70 mm. Hg. The skin and hair were of normal texture. The nasopharynx and tongue appeared normal. There was no glandular enlargement, jaundice, or vascular spiders. Prominent veins coursed over the abdomen and lower thorax. The heart and lungs were normal. There was slight sacral edema but none of the extremities. Neurological, rectal, and vaginal examinations showed no significant changes. Neither liver nor spleen was felt after abdominal paracentesis.

Laboratory findings on admission to the hospital were: Rbc 4.75 million per c. mm.; Wbc 5700 per c. mm.; Hb. 13.4 grams per cent; erythrocyte sedimentation rate 24 mm. per hr.; fasting blood sugar 104 mgm. per cent; serum cholesterol 250 mgm. per cent (ester 189); serum alkaline phosphatase 28 Bodansky units; serum bilirubin 0.4 mgm. per cent; serum albumin 3.1 grams per cent; serum globulin 1.7 grams per cent; non-protein nitrogen 25 mgm. per cent; cephalin flocculation test 4+; thymol turbidity test 3+; prothrombin time 15 sec.; urine urobilinogen 0.24 Ehrlich units per hour; bromsulfalein dye test 35 per cent in 30 min.; urinalysis showed 1+ albumin and increased white cells. Urea clearance 97 per cent of normal; phenolsulfonphthalein test 85 per cent in 2 hours and 10 min.

Three months after entry the edges of the liver and spleen were barely felt. After a nutritious diet and intravenous injections of liver extract she gained in strength and well being but showed no evidence of improved liver function. Ascites required tapping (8000 cc.) every 2 or 3 weeks. Seven months after entry observations were made on the use of intravenous serum albumin. Ten days after the conclusion of these studies the laboratory tests were as follows: Rbc 4.6 million per c. mm.; Wbc 4800 per c. mm.; Hb. 11.1 grams per cent; erythrocyte sedimentation rate 25 mm. per hour; cholesterol 230 mgm. per cent (ester 158); serum bilirubin 0.4 mgm. per cent; cephalin flocculation test 4+; urine urobilinogen 0.3 Ehrlich units per hour; prothrombin time 17 sec.; bromsulfalein dye test 34 per cent in 30 min.; serum albumin 4.2 grams per cent; serum globulin 1.3 grams per cent; non-protein nitrogen 27 mgm. per cent. Urine was negative except for 1+ albumin and increased white cells. The above data show no significant changes from previous determinations except for increased serum albumin.

The patient was transferred 4 months later to the Surgical Service for a "button operation" in order to relieve ascites formation. However, this procedure has been without effect. At the time of operation the liver was observed to be very small and nodular. No biopsy was obtained.

Patient G.

A 42 year old tailor entered the Research Service August 20, 1943, because of abdominal swelling. For 12

years he had been exposed to benzene cleaning fumes. For 5 years he had been drinking excessively of whiskey and beer. His appetite was good and his diet seemed to be adequate in protein foods. For 1 year there had been spells of vomiting in the morning and libido had decreased. After a severe blow on the abdomen, 3 months preceding entry, he noted tarry stools, persisting 3 weeks. Shortly thereafter he was aware of jaundice, abdominal swelling, edema of the ankles, and oliguria. Three weeks before entry he was admitted to the Presbyterian Hospital. After receiving 2 abdominal paracenteses and supportive care the patient was transferred to the Columbia Research Service for further care.

Examination showed an obese, hairy-chested, middle aged man. Weight 200 lbs.; temperature 100° F.; pulse 90 per min.; blood pressure 120/80 mm. Hg. Sclerae were icteric. There were numerous vascular spiders over the arms and posterior thorax. Teeth were carious and the tongue was smooth at the lateral margins. The heart and lungs showed no abnormal findings. The abdomen was greatly extended with bulging flanks, and fluid wave. The firm, non-tender liver was felt one hand's breadth below the costal rim but the spleen was not palpable. There were typical "liver palms" and minimal edema of the ankles. Reflexes were normal except for absent ankle jerks. Vibratory sensation was absent in the toes. The left testicle was atrophied. Painful external and internal hemorrhoids were present.

Laboratory findings at entry were as follows: Rbc 3.54 million per c. mm.; Hb. 13.2 grams per cent; Wbc 8900 per c. mm.; differential smear normal. Urinalysis normal except for +1 albumin. Urea clearance 119 per cent of normal; P.S.P. test 85 per cent in 2 hours 10 min.; plasma albumin 2.6 grams per cent; plasma globulin 4.0 grams per cent; non-protein nitrogen 28 mgm. per cent; bromsulfalein test 68 per cent in 30 min.; icterus index 25; cephalin flocculation reaction 3+; prothrombin time 26 sec.; total cholesterol 184 mgm. per cent; fasting glucose 113 mgm. per cent; BMR +16 and +9.

During the 21 months preceding albumin therapy, the patient had persistent fever (100 to 101° F.), tachycardia, and ascites formation requiring abdominal taps at 7 to 14 day intervals. An umbilical hernia appeared 3 months after entry. Although he showed moderate clinical benefit from dietary care and diuretics there was no change in the rate of ascites formation. A biopsy performed in February 1945 showed changes typical of Laennec's cirrhosis. In June 1945, preceding the albumin therapy the findings were: Rbc 4.3 million per c. mm.; Hb. 13.6 grams per cent; Wbc 3400 per c. mm.; plasma albumin 3.0 grams per cent; plasma globulin 2.4 grams per cent; non-protein nitrogen 29 mgm. per cent; bromsulfalein dye test 40 per cent in 30 min.; serum bilirubin 0.9 mgm. per cent; cephalin flocculation reaction 1+; prothrombin time 22 sec.; total cholesterol 144 mgm. per cent; urine urobilinogen excretion 1.8 Ehrlich units per hour.

In June 1945, 21 months after entry, the patient received 16 daily injections of concentrated albumin (10% solution, 5 grams). No significant change in the clinical state was observed.

peared during or directly after this experimental period. The laboratory data after the period of albumin injections showed no essential changes other than an increase of plasma albumin.

The patient was maintained on the standard therapeutic regimen during the next 10 months during which no significant changes took place. From May 1946 to April 1947 he received, in addition, intravenous liver extract. At the end of this time a slow, steady diuresis took place with the loss of ascites together with laboratory evidence of clinical improvement.

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OSMOTIC FACTORS INFLUENCING THE FORMATION OF ASCITES IN PATIENTS WITH CIRRHOSIS OF THE LIVER

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According to present concepts, the ascites that occurs in patients with cirrhosis of the liver is formed by transudation from the plasma into the peritoneal cavity through the walls of the capillaries of the portal system. A ready explanation for this transudation has been at hand, based upon the hypothesis originally proposed by Starling (1 to 3) that interchange of fluid between blood and tissue spaces is controlled by the balance between capillary blood pressure and osmotic pressure of plasma proteins. In patients with cirrhosis and ascites the portal venous pressure is high (4); the serum albumin concentration (5 to 7) and the serum colloid osmotic pressure (8) are low. The observations of Post and Patek (7) have suggested that the presence or absence of ascites in patients with cirrhosis of the liver is determined chiefly by the serum albumin level.

Recent observations have thrown doubt upon this concept (9, 10). In the course of an investigation of the effects of intravenous injection of concentrated human serum albumin in patients with cirrhosis and ascites (10) it was observed that elevation of the colloid osmotic pressure of the plasma to normal levels for periods of 2 weeks did not decrease the rate of ascites formation. There occurred instead a marked increase in the rate of diffusion of albumin from the plasma into the ascitic fluid and a rise in the colloid osmotic pressure of the ascitic fluid which paralleled that of the plasma.

Because these observations seemed inconsistent with the accepted concepts of the mechanism of ascites formation, the present studies were undertaken in order (a) to determine whether or not the formation of ascites in patients with cirrhosis of the liver is controlled by osmotic factors, in accordance with the Starling hypothesis, and (b) to evaluate the role of the diffusion of plasma proteins into the peritoneal cavity.

According to the Starling hypothesis, the flow of fluid across the portal capillary membrane is

controlled by the balance of hydrostatic and colloid osmotic pressures on both sides of the membrane (11), such that at equilibrium:

portal capillary pressure minus intra-abdominal hydrostatic pressure = plasma colloid osmotic pressure minus ascitic colloid osmotic pressure.

Disturbance of the osmotic balance by alteration of any of these factors should produce corresponding changes in the rate and direction of flow.

In order to test the validity of this hypothesis, direct measurements were made of (1) the plasma colloid osmotic pressure, (2) ascitic colloid osmotic pressure, (3) the intra-abdominal hydrostatic pressure, and (4) the ascitic fluid volume, both in the unmodified course of the formation and loss of ascites, and after experimental alteration of one of the osmotic factors. The experimental elevation of plasma colloid osmotic pressure was effected by (a) mercupurin diuresis and (b) intravenous injection of concentrated human serum albumin. Plasma colloid osmotic pressure was decreased by the oral administration of physiological saline solution. Ascitic colloid osmotic pressure was decreased by the intraperitoneal injection of salt solution. Intra-abdominal hydrostatic pressure was increased by the accumulation of ascites and decreased by abdominal paracentesis. The net transfer of albumin was calculated from the increase or decrease of total ascitic albumin (ascitic albumin concentration \times ascitic volume).

METHODS

The subjects of these studies were 10 patients with cirrhosis of the liver receiving treatment on the wards of the Columbia Research Service at the Goldwater Memorial Hospital. The clinical diagnoses were confirmed by liver biopsy (8 cases), laparotomy (1 case), or autopsy (1 case). Nine cases were of the Laennec type 1 (patient N.) was diagnosed as post-necrotic cirrhosis. The therapeutic regimen consisted, in general, of the nutritional diet and supplements rich in vitamin B complex described by Patek and Post (12).

Colloid osmotic pressure

For the determination of colloid osmotic pressure an Hepp osmometer (13) was used. In this apparatus a 2 cc. sample of serum or ascitic fluid is brought into equilibrium at room temperature with a protein-free "reference" salt solution across a semi-permeable collodion membrane. The colloid osmotic pressure is directly measured as the negative pressure upon the "reference" solution which prevents the motion of a capillary meniscus. The equilibrium is rapidly established (usually within 5 minutes), without significant volume change in the sample and before appreciable loss of carbon dioxide. The reproducibility of successive measurements was found to be ± 1 mm. H₂O (118 duplicate measurements). Comparison of measurements upon the same specimen of serum before and after storage in the refrigerator for several days, and with change of membrane, showed an average difference of 7 mm. H₂O (40 duplicate measurements).

The Hepp osmometer and directions for its use were furnished through the kindness of Dr. Bernard Davis. The composition of the "reference" solution was: 0.14 M NaCl, 0.025 M NaHCO₃. Collodion membranes of suitable permeability were prepared by the method of Pierce (14): 15 cc. of a 2 per cent Parlodion solution in 50 per cent absolute alcohol-50 per cent ether, containing 10.0 volumes per cent ethylene glycol, were poured onto a mercury surface in an evaporating dish of 9.5 cm. diameter, and evaporated to complete dryness (24 hours) in a moisture-free atmosphere. The membranes were washed for 48 hours in running tap water, and stored in distilled water.

Blood samples were taken under standardized conditions in order to minimize the effects of venous stasis (15, 16) and of postural variation (17 to 19). The patients were kept flat in bed for at least 2 hours before the taking of blood samples. Venipuncture was accomplished as rapidly as possible after the tourniquet application, usually within 1 minute. Under these conditions the average diurnal variation of serum colloid osmotic pressure in a given patient was ± 2 per cent (14 samples). On successive days the average variation was ± 4 per cent (59 samples). Specimens of ascitic fluid were obtained without difficulty or complication by inserting a No. 20 needle through the anterior abdominal wall after local infiltration with novocaine.

Ascitic fluid volume

For the measurement of the volume of ascitic fluid a procedure was devised based upon the dye dilution method for the determination of plasma volume (20). The dyes Evans blue (T-1824) and brilliant vital red were found suitable. After an ascitic fluid "blank" was withdrawn, a measured quantity of dye was injected intraperitoneally. Vigorous ballottement of the abdomen with several changes of position produced apparently uniform distribution of the dye in $\frac{1}{2}$ hour. A second specimen of ascitic fluid, obtained $\frac{1}{2}$ to 1 hour after dye injection, was compared with the blank in a spectrophotometer and the volume calculated. Following intraperitoneal injection of

the dye there was no significant decrease in dye concentration in the ascitic fluid within several hours. The dye was first detected in the plasma 24 to 48 hours after intraperitoneal injection.

In order to test the accuracy of the method, dye-volume determinations were performed before and after removal of 4 to 7 liters of fluid from the peritoneal cavity (5 comparisons). The average difference, regardless of sign, between the calculated volume differences and the actual volumes removed was 375 cc.

Intraperitoneal hydrostatic pressure

As a possible index to the effective hydrostatic pressure in the tissues surrounding the portal capillaries, measurements were made of the intraperitoneal hydrostatic pressure under the following standard conditions. With the patient flat on his back, a No. 20 needle was inserted through the lateral abdominal wall at a standard level halfway from back to front. The ascitic fluid was allowed to seek a stable level in an L-tube manometer.

Readings were immediately reproducible within 5 mm. H₂O. In the same patient, in 2 successive periods of ascites accumulation, at equal body weights, manometric values agreed within 10 mm. H₂O.

RESULTS

I. Observations upon the unmodified course of ascites formation

A. The accumulation of ascites between abdominal paracenteses

Repeated cycles of accumulation of ascites between abdominal paracenteses were studied in patients whose ascites formation was at an approximately steady state. The object was to determine what influence the accumulation of ascites has upon the balance between the osmotic forces.

Ascitic fluid volumes were measured at intervals through 10 periods of ascites accumulation in 4 patients. The average interval between abdominal paracenteses was 12 days. The average ascitic volume at paracentesis was 8 liters. The ascitic volume increased rapidly during the first 3 days post-paracentesis; thereafter the rate of increase was uniform. The intraperitoneal hydrostatic pressure increased with the accumulation of ascites from an initial value of about 125 mm. H₂O to about 225 mm. H₂O at the time of paracentesis (2 periods in 1 patient). The colloid osmotic pressures of the serum and ascitic fluid (15 periods in 6 patients) were approximately constant throughout each period (Figure 1).

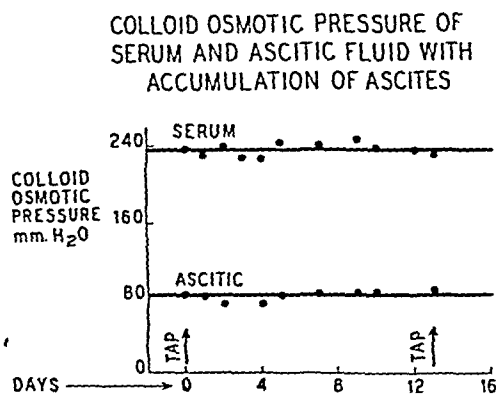


FIG. 1. COLLOID OSMOTIC PRESSURE OF SERUM AND ASCITIC FLUID IN THE COURSE OF ASCITES ACCUMULATION (PATIENT D.)

The constancy of the colloid osmotic pressures of the plasma and ascitic fluid in the face of a nearly 2-fold increase in the intra-abdominal hydrostatic pressure suggests that either (1) transudation into the peritoneal cavity is independent of the intra-abdominal pressure in this range or (2) some type of equilibrium is maintained between the hydrostatic and colloid osmotic pressures as the ascites accumulates. In the latter case, the findings suggest that increase in intra-abdominal hydrostatic pressure in the course of ascites accumulation is balanced by a concomitant increase in portal capillary pressure.

B. Long term studies of the formation and spontaneous absorption of ascites

The measurements of colloid osmotic pressure, described above in patients at a steady state, were extended over longer periods of time in order to determine the relation between the colloid osmotic pressures of the plasma and ascitic fluid in the course of (1) spontaneous variation in the plasma colloid osmotic pressure and (2) spontaneous absorption of ascites.

Three patients were studied through periods of 4 months to 1 year. Changes in serum colloid osmotic pressure (up to 120 mm. H_2O) were accompanied by parallel changes in ascitic colloid osmotic pressure, so that the difference between the colloid osmotic pressures of the serum and ascitic fluid remained approximately constant (± 10 mm. H_2O) in each patient throughout the periods of study.

In 2 of these patients the studies extended through periods both of formation and spontaneous loss of ascites. Loss of ascites in these cases was gradual over 2 to 3 months. Associated with the diuresis there was a gradual elevation of the serum colloid osmotic pressure from levels of 200 to 240 mm. H_2O to levels of 270 to 300 mm. H_2O . The colloid osmotic pressure of the ascitic fluid increased with that of the serum. Associated with the transition from formation to absorption of ascites there was no change in the relation between the colloid osmotic pressures of the serum and ascitic fluid.

These observations suggest that in each patient a constant difference between the colloid osmotic pressures of the plasma and ascitic fluid is maintained through equilibrium with a constant effective hydrostatic pressure in the portal system. This phenomenon suggests that there is no significant decrease in effective portal pressure during spontaneous loss of ascites.

II. Experimental alteration of the osmotic factors

In order to test the hypothesis that approximate equilibrium exists between the plasma and ascitic fluid, the colloid osmotic pressures of the plasma and ascitic fluid were experimentally altered by the following measures: (A) mercupurin diuresis, (B) oral administration of isotonic sodium chloride solution, (C) intraperitoneal injection of isotonic sodium chloride solution, and (D) intravenous injection of concentrated human serum albumin.

A. Mercupurin diuresis

Mercupurin was used as a means of increasing the colloid osmotic pressure of the plasma. Previous authors have reported elevation of the serum colloid osmotic pressure (21) or of the serum protein concentration (22) in association with organic mercurial diuresis, presumably through the mechanism of hemoconcentration. Mercurial diuresis without apparent hemoconcentration has been observed (22, 23, 24). In the present studies, significant elevation of serum colloid osmotic pressure occurred when the urinary output exceeded the control values by 2500 cc. (4 experiments). The colloid osmotic pressure rose from 15 to 20 mm. H_2O , 4 hours after intravenous injection

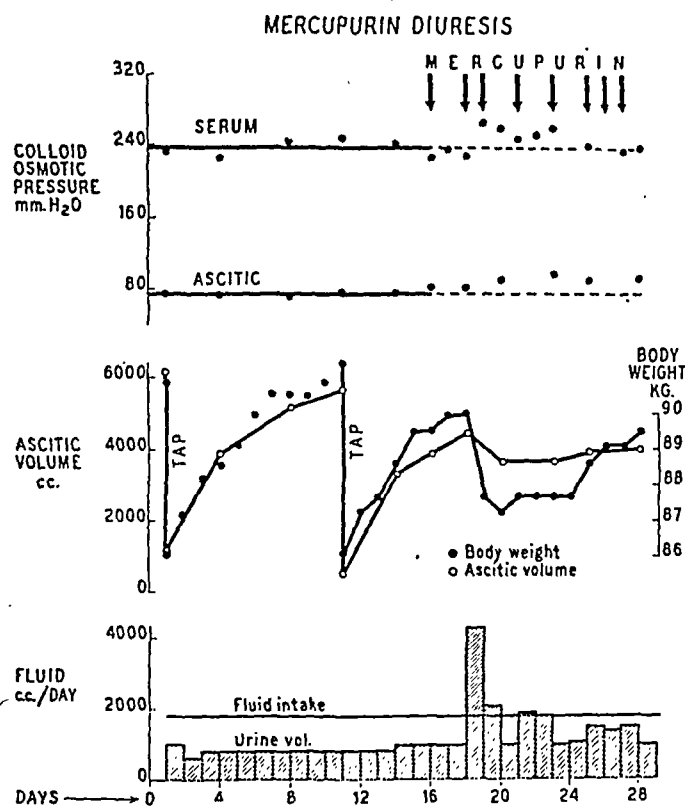


FIG. 2. EFFECTS OF REPEATED INTRAVENOUS INJECTIONS OF MERCUPURIN (PATIENT G.)

Mercupurin injections (2 cc.) are indicated by arrows at the top of the figure.

of 2 cc. mercupurin, reached a peak increase of 30 to 40 mm. by the 8th hour, and then fell about 15 mm. H₂O in the next 16 hours. In 4 cases where excess urinary output was less than 2000 cc., no significant elevation of serum colloid osmotic pressure was observed.

The effects of repeated daily intravenous injections of mercupurin (2 cc.) were studied in 3 experiments upon 2 patients through periods of 5 to 11 days (Figure 2). The diuretic response was variable, with extremes of 0 to 3500 cc. per 24 hours. The serum colloid osmotic pressure, measured 24 hours after injection, increased only slightly during the period of mercupurin injections. The body weight and ascitic volume decreased following diuresis. The colloid osmotic pressure of the ascitic fluid increased by about the same increment as that of the serum, with a lag of 24 to 48 hours.

B. Oral administration of isotonic sodium chloride solution

In the present investigation, salt solution was administered in order to effect hemodilution and

decrease of the plasma colloid osmotic pressure (25, 26). Two patients were given orally, in addition to their usual salt-poor regimen, 2000 cc. of 0.9 per cent sodium chloride daily for 3 successive days (Figure 3). The serum colloid osmotic pressure fell progressively. The maximum decrease was 40 mm. H₂O. The body weight and ascitic volume increased precipitously by comparison with the control periods. The excess ascitic fluid represented approximately $\frac{1}{2}$ the increase in body weight. The colloid osmotic pressure of the ascitic fluid decreased with that of the serum. The maximum decrease was 30 mm. H₂O.

C. Intraperitoneal injection of isotonic sodium chloride solution

In order to study the effects of decrease of the colloid osmotic pressure of the ascitic fluid, the ascitic fluid was diluted by withdrawing ascitic fluid from the peritoneal cavity and instilling an equal volume of isotonic sodium chloride solution (2 cases). Strict precautions were exercised

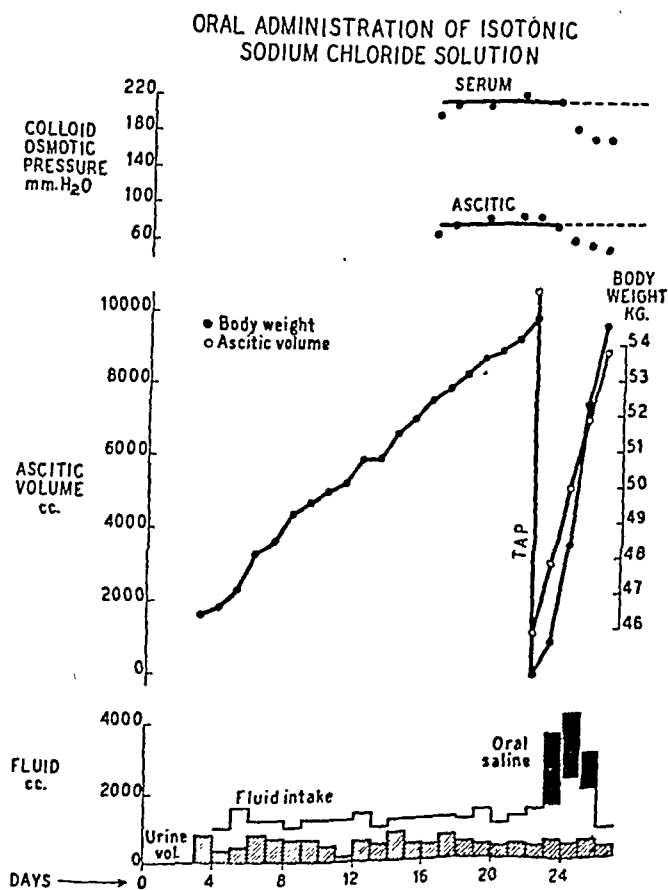


FIG. 3. ORAL ADMINISTRATION OF ISOTONIC SODIUM CHLORIDE SOLUTION (PATIENT N.)

against bacterial or chemical inflammation of the peritoneum.

The results of repeated daily intraperitoneal instillations of salt solution are shown in Figure 4. The colloid osmotic pressure of the ascitic fluid was decreased step-wise to a minimum value 80 mm. H_2O below the preinjection level. In the 6 days following the last intraperitoneal injection, the ascitic colloid osmotic pressure rose to a level 50 mm. H_2O below the control level. The rate of ascites formation showed a slight decrease. The serum colloid osmotic pressure decreased gradually during the period of dilution of the ascitic fluid. The final decrease (50 mm. H_2O) was approximately the same as that of the colloid osmotic pressure of the ascitic fluid.

The decrease in plasma colloid osmotic pressure was due, at least in part, to an increased transfer of protein from plasma to ascitic fluid. During the 10-day control period the calculated total ascitic fluid albumin increased 6 grams per day. During the first 8 days of ascitic fluid dilution, the calculated average daily increase was 10 grams. Since there were neither physical signs nor cytological evidence of peritoneal inflammation, it

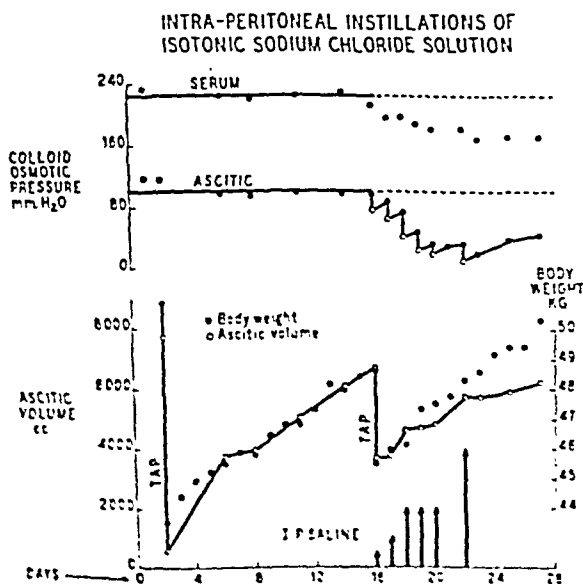


FIG. 4. EFFECTS OF REPEATED DILUTION OF ASCITIC FLUID BY INTRA-PERITONEAL INSTILLATIONS OF ISOTONIC SODIUM CHLORIDE SOLUTION (PATIENT N.)

Ascitic fluid was withdrawn and an equal volume of salt solution substituted. Arrows represent volumes of 500 cc., 1000 cc., 2000 cc., and 4000 cc.

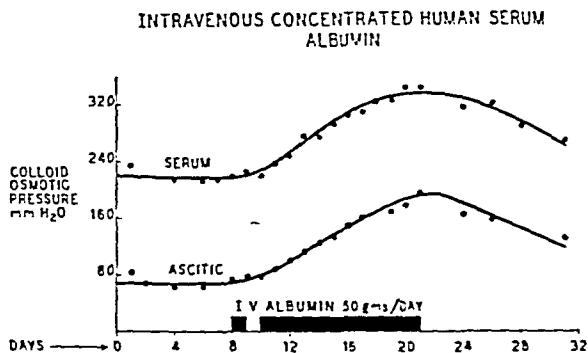


FIG. 5. EFFECTS OF DAILY INTRAVENOUS INJECTION OF CONCENTRATED HUMAN SERUM ALBUMIN (PATIENT N.)

may be postulated that the diffusion of protein was increased as a function of an increased protein concentration gradient between the plasma and the ascitic fluid.

D. Intravenous injection of concentrated human serum albumin

The effects of intravenous administration of concentrated albumin are described in detail in a separate report (10). From the point of view of the present study, the results may be summarized as follows:

Three patients received daily intravenous injections of 50 grams of concentrated human serum albumin (containing 0.3M NaCl) for periods of 13 to 16 days. The serum colloid osmotic pressure increased during the period of albumin therapy and then decreased (Figure 5). The values reached normal levels (above 300 mm. H_2O) by the 7th day of injection and remained in the normal range for 2 weeks. The colloid osmotic pressure of the ascitic fluid increased and decreased with that of the serum (Figure 5). There was a 3-fold increase in the rate of transfer of albumin from the plasma to the ascitic fluid during the period of albumin therapy. The rate of formation of ascites was not decreased.

DISCUSSION

The aims of the present investigation, as outlined earlier, were (1) to determine whether the formation of ascites is controlled by osmotic forces, and (2) to evaluate the role of protein diffusion from the plasma into the ascitic fluid. The ex-

perimental results may be interpreted to throw light upon these problems.

I. Osmotic equilibrium between the plasma and the ascitic fluid

The experimental data suggest that approximate osmotic equilibrium exists between the plasma and the ascitic fluid. In each patient there was a constant difference between the colloid osmotic pressures of the plasma and ascitic fluid. Experimental disturbance of this relation altered the rates of fluid and protein transfer in directions tending to reestablish the previous colloid osmotic pressure difference. It may be postulated that this constancy of the colloid osmotic pressure difference (plasma colloid osmotic pressure minus ascitic colloid osmotic pressure) is maintained through osmotic equilibrium with a constant net hydrostatic force (portal capillary pressure minus intra-abdominal hydrostatic pressure) in accordance with the Starling equilibrium equation. The effective portal pressure in each patient may be assumed to depend upon the degree of intra-hepatic portal obstruction.

As a corollary to this hypothesis, the possibility suggested itself that measurements of the colloid osmotic pressure difference between the plasma and ascitic fluid might serve as an index to the relative portal venous pressure. Preliminary comparisons of such measurements in patients with cirrhosis of the liver, portal venous thrombosis, chronic glomerulonephritis in the nephrotic phase, and peritoneal carcinomatosis tend to support this view (Table I), but comparisons with direct

measurements of the portal venous pressure at laparotomy are necessary for confirmation.

II. Diffusion of plasma proteins into the peritoneal cavity

If the above hypothesis is accepted, that the plasma and ascitic fluid are in osmotic equilibrium, it must follow that transudation into the peritoneal cavity proceeds only by virtue of protein diffusion from the plasma into the ascitic fluid. At a state of true osmotic equilibrium no net transfer of protein or fluid would occur in either direction. But the process of ascites formation may be pictured as one of partial or dynamic equilibrium in the sense that the diffusion of plasma proteins into the ascitic fluid, which would tend to increase the colloid osmotic pressure of the ascitic fluid, is balanced by fluid adjustments sufficiently rapid to maintain osmotic equilibrium. In support of such a concept are the observations of Loeb, Atchley, and Palmer (27), Van Slyke, Wu, and McLean (28), and of Hastings and his co-workers (29), that the electrolyte distribution ratios between the plasma and the ascitic fluid are in accord with the Gibbs-Donnan law (30, 31) governing osmotic equilibrium between solutions of unequal protein concentration.

The influence of protein diffusion into the ascitic fluid is illustrated in 2 experiments described above. The effects of mercupurin diuresis and of intravenous injection of concentrated albumin are similar in that (1) the plasma colloid osmotic pressure is increased in both instances, and (2) the ascitic colloid osmotic pressure tends to increase parallel with that of the plasma. However, in the former experiment, the increase of ascitic colloid osmotic pressure is apparently effected by absorption of fluid from the peritoneal cavity, while, in the latter, there occurs instead an increased diffusion of albumin from plasma to ascitic fluid.

This increased diffusion of albumin into the ascitic fluid following intravenous injection of albumin may be correlated with the observed increase in plasma volume (10). Previous workers have shown that (a) experimental elevation of blood volume produces increased portal venous pressure (1), and (b) experimental venous congestion produces increased diffusion of plasma proteins through the capillary walls (16). Accord-

TABLE I

Colloid osmotic pressure differences between serum and ascitic fluid in various diseases: Correlation with expected degree of portal obstruction

Disease	Serum C.O.P.	Serum C.O.P. minus ascitic C.O.P.
	mm. H ₂ O	mm. H ₂ O
Laennec's cirrhosis (14 cases)	180-317	175±30 mm.
Portal venous thrombosis (1 case)	275-295	244±5 mm.
Chronic glomerulonephritis in nephrotic phase (2 cases)	87-129	86±6 mm.
Peritoneal tumors	242-310	110±20 mm.
Ovarian carc.: 1		
Breast carc.: 1		
Lymphosarcoma: 1		

ingly, the injection of concentrated albumin may be pictured as having 2 competing effects: (1) elevation of the plasma colloid osmotic pressure, which would tend to withdraw fluid from the peritoneal cavity, and (2) increase of the plasma volume (by rapid absorption of fluid from the interstitial spaces), which would tend to increase the portal capillary pressure and thus increase the rate of diffusion of albumin into the ascitic fluid. The net effect in a given patient might be expected to depend upon (a) the permeability of the portal capillaries, (b) the renal response to an increase of plasma volume (10), and (c) the amount of albumin injected.

III. *The mechanism of ascites formation*

The pathogenesis of ascites in patients with cirrhosis of the liver has not been revealed in the present studies. It would appear that ascites formation is the result of a combination of factors: increased portal capillary pressure, decreased plasma colloid osmotic pressure, and increased diffusion of protein through the walls of the portal capillaries. These in turn may be influenced by such factors as: increased plasma volume (10, 32), electrolyte changes, and antidiuretic substances (9, 10). The relationships among these several factors need further study.

SUMMARY

Osmotic factors influencing the formation of ascites were studied in 10 patients with cirrhosis of the liver. The colloid osmotic pressure of serum and ascitic fluid, the intraperitoneal hydrostatic pressure, and ascitic volume were measured (a) during the unmodified course of formation and loss of ascites, and (b) following experimental alteration of the colloid osmotic pressure of the plasma or ascitic fluid.

1. In the unmodified course of formation and loss of ascites, changes in serum colloid osmotic pressure were accompanied by parallel changes in ascitic colloid osmotic pressure, so that the difference between the colloid osmotic pressures of the serum and ascitic fluid was approximately constant in each patient through periods of 4 months to 1 year.

2. The accumulation of ascites between abdominal paracenteses was associated with a nearly 2-

fold increase in the mean intraperitoneal hydrostatic pressure. Except for a brief period immediately following paracentesis, the ascitic volume increased at a uniform rate. The colloid osmotic pressures of the serum and ascitic fluid remained constant.

3. Increase of the colloid osmotic pressure of the plasma following mercupurin diuresis was associated with: (a) absorption of fluid from the peritoneal cavity, and (b) increase of the colloid osmotic pressure of the ascitic fluid.

4. Decrease of the colloid osmotic pressure of the plasma following the oral administration of isotonic sodium chloride solution was associated with: (a) increased transudation into the peritoneal cavity, and (b) decrease of the colloid osmotic pressure of the ascitic fluid.

5. Decrease of the colloid osmotic pressure of the ascitic fluid by the intraperitoneal injection of isotonic sodium chloride solution was followed by: (a) a slight decrease in the rate of ascites formation, (b) increased diffusion of plasma proteins into the ascitic fluid, and (c) decrease of the plasma colloid osmotic pressure.

6. Increase of the colloid osmotic pressure of the plasma by the intravenous injection of concentrated human serum albumin in 3 patients did not result in absorption of fluid from the peritoneal cavity. The effects were: (a) a 3-fold increase in the rate of transfer of albumin from the plasma into the ascitic fluid, and (b) increase of the colloid osmotic pressure of the ascitic fluid parallel with that of the plasma.

7. In each instance, the changes in the rates of fluid and protein exchange tended to restore the preexisting constant difference between the colloid osmotic pressures of the plasma and ascitic fluid.

CONCLUSIONS

1. Approximate osmotic equilibrium exists between the plasma and the ascitic fluid during unmodified formation and loss of ascites.

2. The flow of fluid between the plasma and ascitic fluid is influenced by experimental disturbance of the osmotic balance, in accordance with the Starling hypothesis.

3. The diffusion of plasma protein into the ascitic fluid is an important factor influencing the

formation of ascites, both in the unmodified course and after experimental alteration of the osmotic balance.

ACKNOWLEDGMENTS

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VARIATIONS IN THE BLOOD PRESSURE RESPONSE TO REPEATED ADMINISTRATION OF TETRAETHYL AMMONIUM CHLORIDE¹

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It has been demonstrated by a number of investigators that temporary pharmacological denervation of the autonomic nervous system may be produced through the use of the tetraethyl ammonium ion by means of its action at the autonomic ganglia (1 to 5). In the course of experiments utilizing the tetraethyl ammonium ion in the study of neurogenic mechanisms in essential hypertension, variation in blood pressure response to this drug both in a given individual and in different individuals suggested the necessity of determining whether increasing tolerance may develop during the test period and whether the basic tone (due to humoral and other intrinsic factors) of the denervated arterial vascular tree is constant or varying. The results of numerous serial intravenous injections of tetraethyl ammonium chloride in 6 hypertensive patients are presented as a preliminary answer to these questions.

After entering the laboratory, these patients rested 30 minutes or more in the horizontal position before 5 baseline blood pressure readings were made at minute intervals. Four cubic centimeters (400 mgm.) of tetraethyl ammonium chloride² were then injected into an arm vein and blood pressure readings made at 30-second intervals for 5 minutes and thereafter at minute intervals for an additional 5 minutes. The mean of the pressure readings made before the injection was taken as the baseline, the lowest systolic-diastolic reading made after the injection was taken as the endpoint (tetraethyl ammonium chloride floor). This procedure was repeated serially with each patient at intervals of approximately 24 hours for 7 to 15 days.

Three hospital patients and 3 ambulatory patients were studied. Two of the patients were in

the malignant phase of the disease and 4 in the benign phase. The results are shown in Figures 1 to 3.

No tendency to develop a resistance to the depressor action of the drug was noted in any of the patients during the period of testing. There was, however, considerable day to day variation in both the magnitude of the depressor response and of the blood pressure "floor" reached. The minimum variation of the depressor response (80/44 to 45/

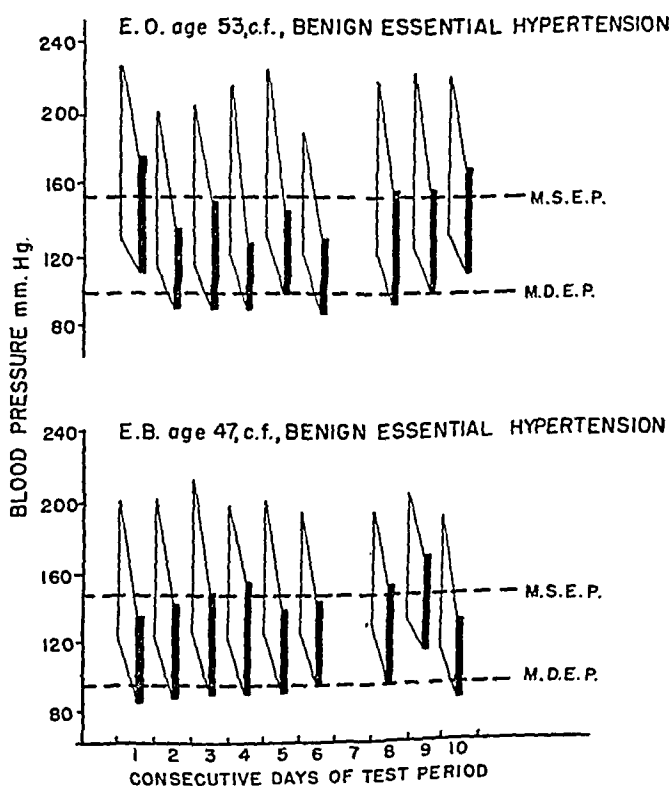


FIG. 1. CONSECUTIVE DEPRESSOR RESPONSES TO TEAC (TETRAETHYL AMMONIUM CHLORIDE)

Light vertical lines connect control systolic and diastolic blood pressures. Heavy vertical lines connect systolic and diastolic pressures at height of TEAC effect (TEAC floor). The 2 horizontal broken lines represent the mean systolic and diastolic endpoints (TEAC floor) of all determinations. Note the day to day variation in the TEAC floor and the lack of any evidence of increasing tolerance to the drug.

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² Etamon chloride supplied by Parke, Davis & Company through the courtesy of Dr. E. C. Vonder Heide.

30 mm. Hg) occurred in patient E. H. (Figure 2), the maximum (82/71 to 18/1 mm. Hg) in patient

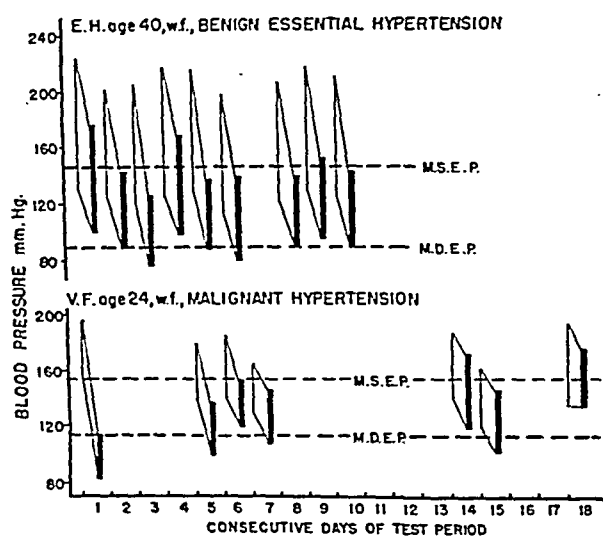


FIG. 2. See legend, Figure 1

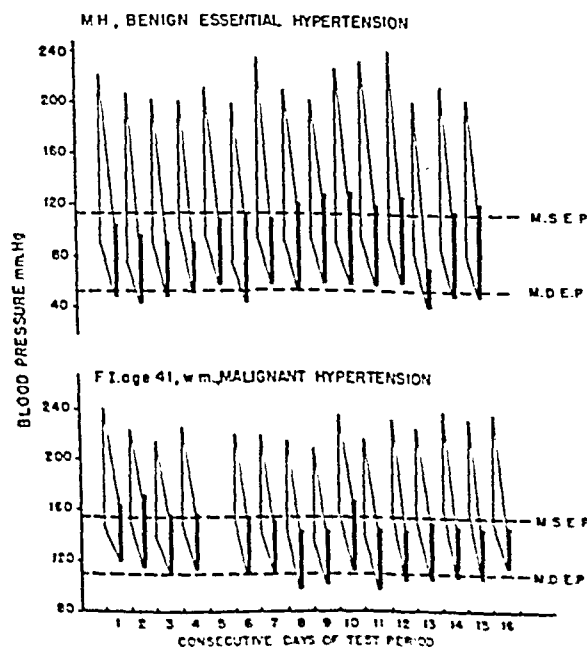


FIG. 3. See legend, Figure 1

V. F. (Figure 2). The fluctuations in the blood pressure floor varied from 170/114 to 144/96 mm. Hg in patient F. I. (Figure 3) to 176/135 to 114/84 mm. Hg in patient V. F. (Figure 2). No correlation could be demonstrated between variations in the initial height of blood pressure and the floor levels reached after tetraethyl ammonium chloride.

SUMMARY

Serial intravenous injections of tetraethyl ammonium chloride in 6 hypertensive patients revealed in all cases considerable daily fluctuation in both the magnitude of depressor response and the blood pressure floor. There was no evidence of the development of increasing tolerance to the depressor effect of the drug on repeated administration. For this reason, the data suggest that fluctuating humoral and neurogenic mechanisms interact as factors in clinical hypertension.

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THE NATURE OF THE COLD PRESSOR TEST AND ITS SIGNIFICANCE IN RELATION TO NEUROGENIC AND HUMORAL MECHANISMS IN HYPERTENSION¹

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INTRODUCTION

Recent emphasis on the use of partial sympathectomy in the treatment of essential hypertension has focused attention on the "neurogenic component" in this disease. That the autonomic nervous system plays a role cannot be questioned; however, the physiologic basis for most operative procedures in hypertension has not been clearly established. Humoral pressor mechanisms have also been emphasized as being of primary significance in its pathogenesis but, likewise, their role in clinical hypertension is not clear.

It becomes, then, a matter of great importance for the further understanding of this disease to develop methods which will make it possible to identify at any given time the presence, extent, and character of autonomic vasomotor activity. Such methods, in addition to providing information regarding the nature of neurogenic mechanisms, should at the same time help to evaluate non-nervous factors and their inter-relationships with vegetative nervous control. In a study of blood pressure levels (1) utilizing differential methods for partial and complete sympathetic denervation, it was found that in a majority of cases of hypertension there apparently is a direct quantitative relationship between the level of the blood pressure and the anatomical extent of the arteriolar bed under active vasomotor control. The implications with regard to reduction of blood pressure by partial sympathectomy were discussed.

The present study concerns itself with the effector mechanism of the cold pressor response in hypertension, utilizing varying degrees of sympathetic block induced by spinal anesthesia and tetraethyl ammonium chloride (TEAC). Although, as pointed out elsewhere (2), the clinical value of

this test is limited, the evidence to be presented suggests that its utilization together with other procedures may be useful in evaluating the relative importance of neurogenic and humoral components in hypertension.

MATERIAL AND METHODS

Twenty hypertensive patients are included in this study, 15 having benign essential hypertension, 2, malignant hypertension, 2, polycystic kidneys and 1, acute glomerulonephritis. TEAC² in doses of 3 to 5 cc. (300 to 500 mgm.) administered intravenously was used to induce total sympathetic block (3). Partial sympathetic block was induced by means of spinal anesthesia to varying spinal levels and in 1 additional case, by partial surgical sympathectomy. The cold pressor test (4) was performed in the usual manner, blood pressure being measured after the ½- and 1-minute intervals of immersion of the hand in water at 4° C. (the "cold minute" response). In addition blood pressure measurements were continued after removal of the hand from cold water in order to detect delayed pressor effects.

The procedure was to obtain a control cold pressor response after the subject had rested for ½ hour and after pre-test blood pressure levels had been determined at 1-minute intervals for at least 5 minutes or until a stable base line level was obtained. Soon after the control cold pressor test, TEAC was administered to 20 cases and the cold pressor test repeated during the second and third minutes after injection of TEAC, blood pressures being measured frequently throughout the period of TEAC action. Because of the rapid change in blood pressure in those subjects who showed a depressor response to TEAC, the "cold minute" period sometimes coincided with either the downswing or upswing of the TEAC response which in these and other patients has exhibited a consistent pattern.

In 8 cases the cold pressor response was again tested after high spinal anesthesia and in 5, it was repeated as the anesthesia receded to lower levels. In 5 cases, after the response of the cold pressor test to spinal anesthesia was determined, the subjects were given TEAC and the cold pressor test again repeated in the usual manner.

In an additional case of essential hypertension not included in the above series, 2 control cold pressor tests

¹ The work described in this paper was aided in part by a grant from the Psychosomatic Research Fund of the National Committee for Mental Hygiene, Inc.

² Etamon chloride supplied by Parke, Davis and Company through the courtesy of Dr. E. C. Vonder Heide.

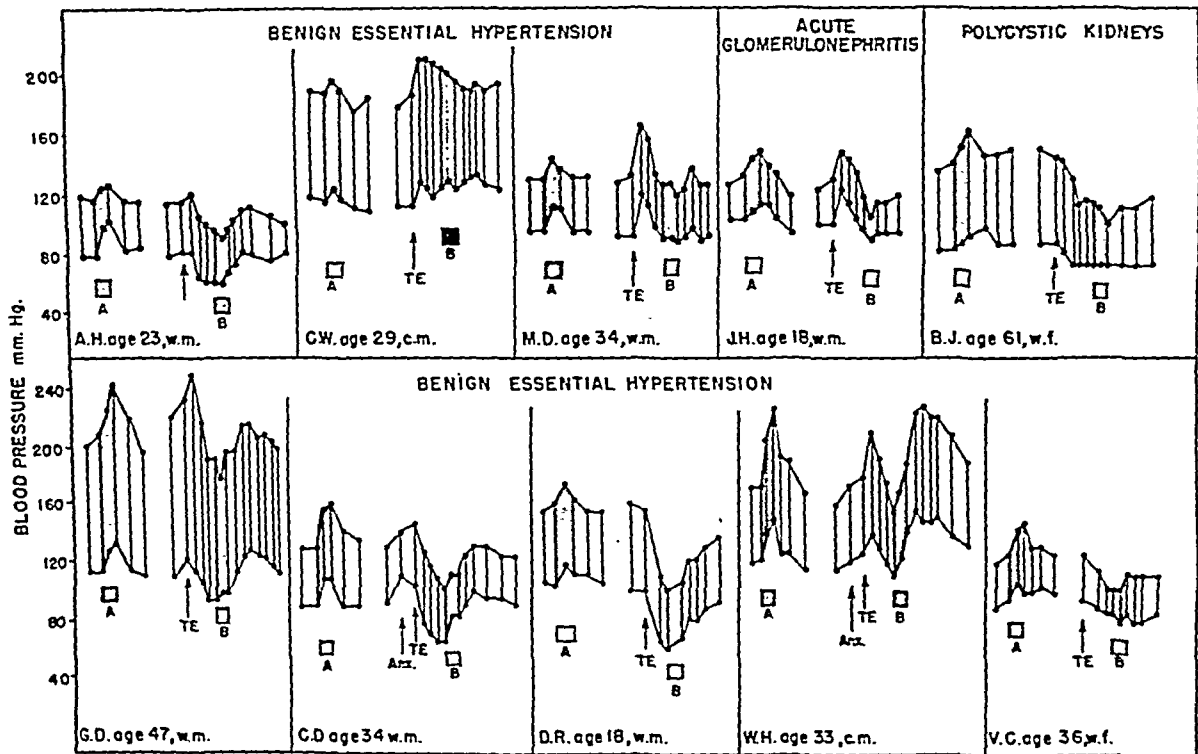


FIG. 1. THE EFFECT ON THE COLD PRESSOR RESPONSE OF PHARMACOLOGIC AUTONOMIC BLOCKADE PRODUCED BY THE INTRAVENOUS ADMINISTRATION OF TEAC

The short intervals represent 30-second readings, the long intervals minute readings of the blood pressure. The cold minute in each instance is identified by the gray stippling. "A" represents the control cold pressor reaction; "B" the cold pressor reaction during the action of TEAC. Note that in all instances after the administration of TEAC (TE) the cold minute reaction is eliminated and that the cold minute exposure in no way alters the curve of the TEAC reaction in those cases where a depressor response is produced by the drug. "Anx." denotes the appearance of overt anxiety. Note the poor depressor response and marked delayed cold pressor rise in patient W. H., who showed marked anxiety prior to injection of TEAC.

were done before sympathectomy and 4 months post-operatively the response was again tested before and during the action of TEAC.

RESULTS

A. Effects of blockade of the autonomic ganglia by the intravenous administration of TEAC:

(1) *Upon the level of the blood pressure:* In 14 cases (70 per cent) the blood pressure was significantly lowered, to normal in 10, near normal in 1 and to intermediate levels in 3. The lowest values were usually obtained between the second and third minutes following the injection. Thereafter, the blood pressure showed a steady upward trend. Six patients (30 per cent) failed to obtain depressor responses and it should be noted that each of these responded to TEAC with a definite immediate rise in blood pressure which reached its

peak at 30 seconds following the injection and lasted 1 to 1½ minutes.³ This rise should be distinguished from the transient rise seen in patient E. H. (Figure 2) which was due to anxiety and pain connected with the venepuncture. Within 30 seconds it was followed by a typical depressor response to the injection of TEAC.

(2) *Upon the cold pressor response:* Two phases of blood pressure response to cold were noted. First, the response which occurred during the minute of exposure to cold ("cold minute"), the classical cold pressor response, and second,

³ In this particular series this immediate rise in blood pressure happened to occur in all of the patients who failed to obtain depressor responses. This is not invariably the case, however, as in other patients who have failed to obtain depressor responses to TEAC, the immediate rise has not been noted.

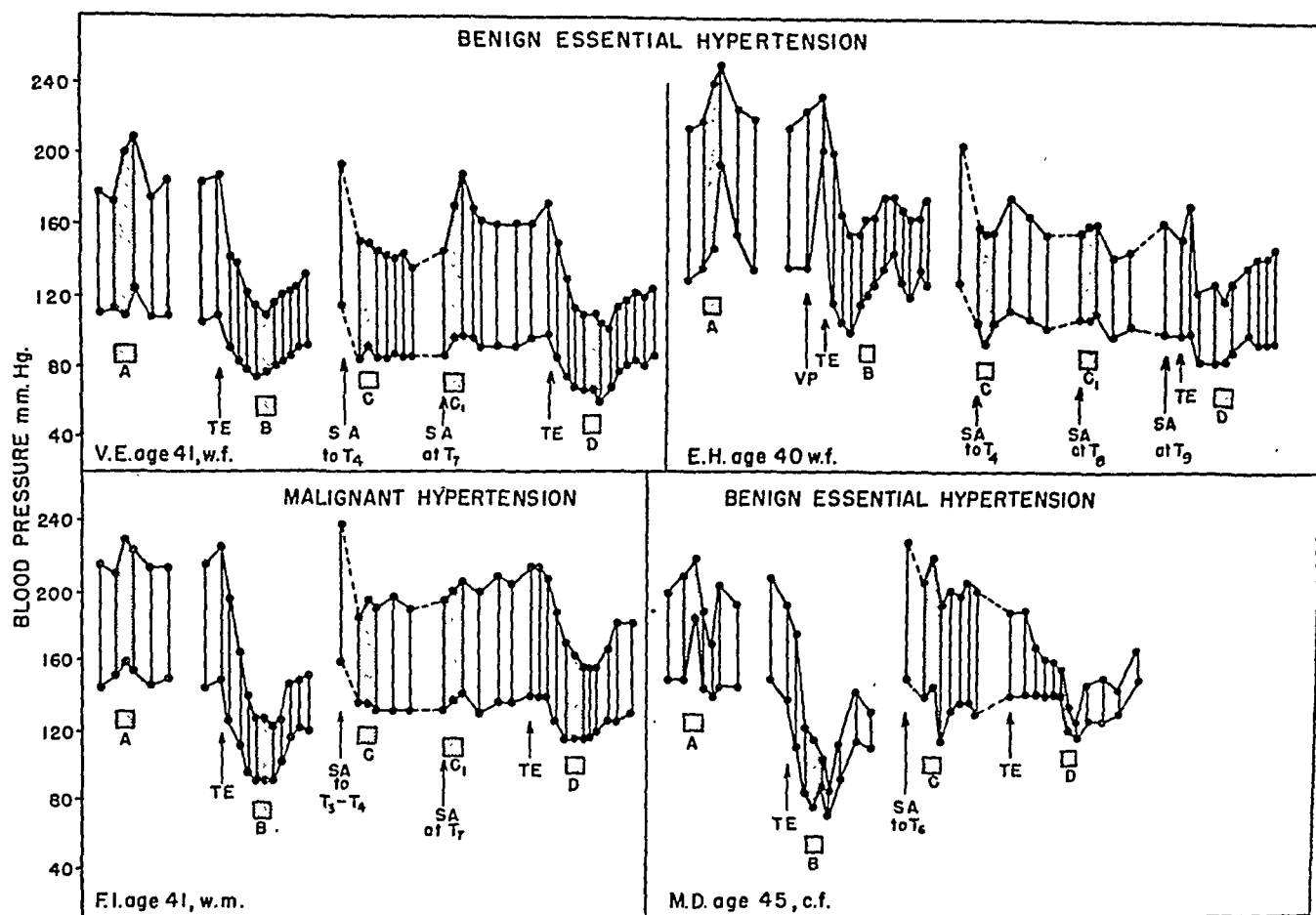


FIG. 2. THE EFFECT ON THE COLD PRESSOR RESPONSE OF VARYING DEGREES OF AUTONOMIC BLOCKADE

The time intervals are denoted in the same manner as in Figure 1. Cold minute exposures are denoted as follows: A—Control. B—during the action of TEAC. C—under high spinal anesthesia (SA). C₁—under spinal anesthesia at lower level. D—after administration of TEAC (TE) during the period of spinal anesthesia. VP denotes venepuncture. Note the complete abolition of the cold minute response during the period of TEAC effect and the varying degrees of dampening of this reaction at different levels of spinal anesthesia. Fall in blood pressure in response to cold exposure under conditions of autonomic blockade seen in patient M.D. is characterized by narrowing of the pulse pressure and is felt to be a manifestation of venous pooling.

delayed pressor responses which occurred after the hand had been removed from cold water.

In all cases TEAC abolished the cold minute pressor response irrespective of the type of hypertension, the degree of depressor effect of the drug, or of the type of control cold pressor reaction. In those cases in which the blood pressure fell in response to TEAC the cold minute exposure did not significantly influence the character of the TEAC response, whether the exposure occurred during the downswing, floor, or upswing of the blood pressure curve (Figures 1, 2, and 3).

In 1 case in which the cold minute occurred during the downswing of the TEAC blood pressure reaction, a very sharp transient rise in pressure occurred immediately after removal of the hand from cold water (Figure 4). This patient

had had a delayed recovery period during his control cold pressor test and reacted with a great deal of anxiety and protest to the discomfort of the cold pain. Similar but much less marked delayed transient elevations in pressure following the cold pressor test performed under these conditions of autonomic block were seen in 11 other patients. There was no correlation between the occurrence of this delayed phenomenon and the patient's reaction to the drug in terms of modification of blood pressure level.

B. Comparison of the effects on the cold pressor response of total autonomic blockade (TEAC) with those of partial sympathetic denervation (high spinal anesthesia). (Table I).

A level of T₄ or slightly above was attained in 7 instances. In all but 1 the cold pressor response

was eliminated at this level. In 5 of these the response was again tested and found to be active (partially in 4, completely in 1) when the anesthesia had receded to lower levels of T7 to T8.

In 5 cases which showed an active cold pressor response at levels of T6 to T8 the response was again eliminated by the intravenous administration of TEAC during the period of anesthesia (Figure 5).

In the patient tested 4 months after partial sympathectomy (analogous to partial denervation by spinal anesthesia) the cold pressor response was found to have remained fully active. TEAC given intravenously then abolished this response.

DISCUSSION

Wolf and Hardy have demonstrated that the cold pressor reaction is initiated by a particular

type of pain termed "cold pain," and that the afferent limb of the reflex is mediated over nervous pathways (5). The mechanism of the effector phase has remained the subject of speculation.

It has been amply demonstrated that TEAC administered in adequate doses effects a transient autonomic block, and that the site of action is at the autonomic ganglia (3). The action of substances such as adrenalin or angiotonin which act upon the neuro-effector apparatus at a point peripheral to the ganglia is not eliminated by this drug (3). It follows then that phenomena mediated through the autonomic nervous system will be abolished by the action of TEAC whereas effects caused by certain humoral mechanisms will remain. Examination of the results of this study indicates that the effector phase of the cold pressor reaction is neurogenic. In all instances the re-

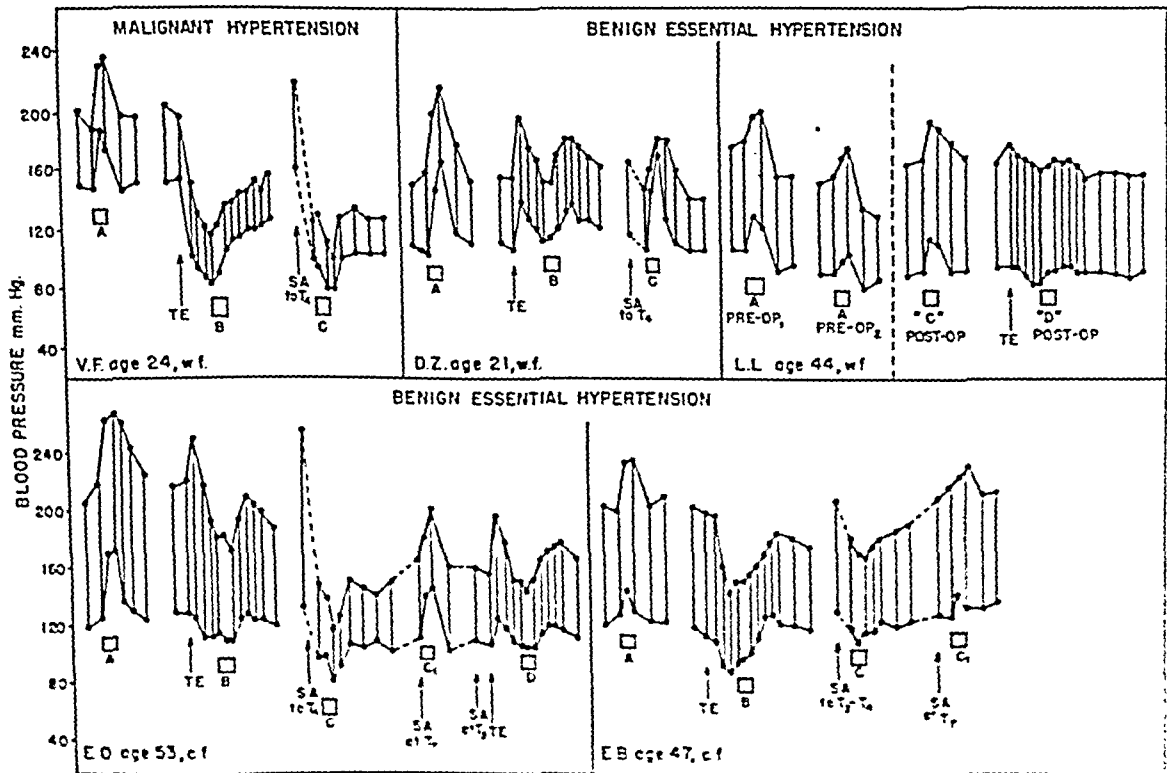


FIG. 3. THE EFFECT ON THE COLD PRESSOR RESPONSE OF VARYING DEGREES OF AUTONOMIC BLOCKADE.

The scheme of presentation is the same as that in Figures 1 and 2. Note again the abolition of the cold pressor response by TEAC (TE) action and the varying degrees of dampening of the response at different levels of spinal anesthesia. In patient L.L., "C" (post-operative) designates the cold pressor reaction 4 months after partial sympathectomy (analogous to spinal anesthesia) and "D" (post-operative) represents the effect of TEAC on the post-operative response. Fall in pressure in response to cold exposure during spinal anesthesia in patients V.F. and E.O. is felt to be a manifestation of venous pooling.

E.H. age 66, w.m., BENIGN ESSENTIAL HYPERTENSION

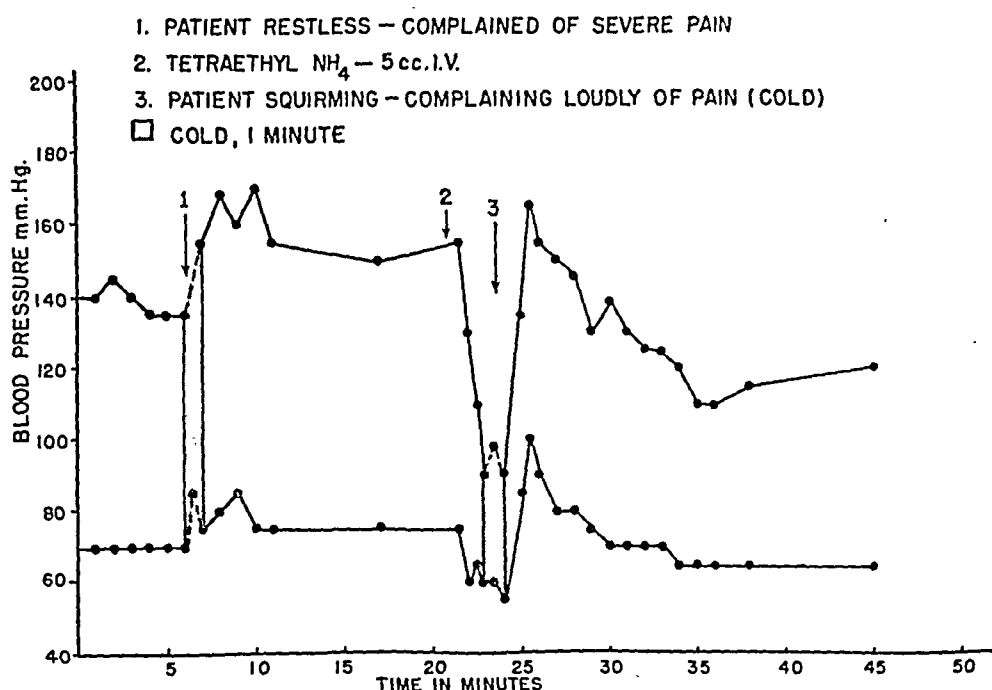


FIG. 4. THE COLD PRESSOR REACTION IN A PATIENT BEFORE AND AFTER THE ADMINISTRATION OF TEAC (TE)

Note the patient's marked subjective response to the experiencing of cold pain, the continuing rise of pressure and delayed recovery after the control cold minute, and the corresponding marked delayed pressor response following cold exposure after TEAC even though the cold minute response was eliminated.

TABLE I

Effect of varying degrees of sympathetic block on cold pressor response

Patient	Diagnosis*	Effect on cold pressor response of TEAC alone	Spinal anesthesia		Effect on cold pressor response of adding TEAC to spinal anesthesia (at a level at which the response is still active)
			Level	Effect on cold pressor response	
1. V. F.	M. H.	Eliminated	T4	Eliminated	
2. F. I.	M. H.	Eliminated	T3-4 T7	Eliminated Dampened	Eliminated
3. M. D.	B. H.	Eliminated	T6	Dampened	Eliminated
4. V. E.	B. H.	Eliminated	T4 T7	Eliminated Unaffected	Eliminated
5. E. H.	B. H.	Eliminated	T4 T8	Eliminated Dampened	Eliminated
6. D. Z.	B. H.	Eliminated	T4	Unaffected	
7. E. O.	B. H.	Eliminated	T4 T7	Eliminated Dampened	Eliminated
8. E. B.	B. H.	Eliminated	T3-4 T7	Eliminated Dampened	

* B. H.—Benign essential hypertension.

M. H.—Malignant hypertension.

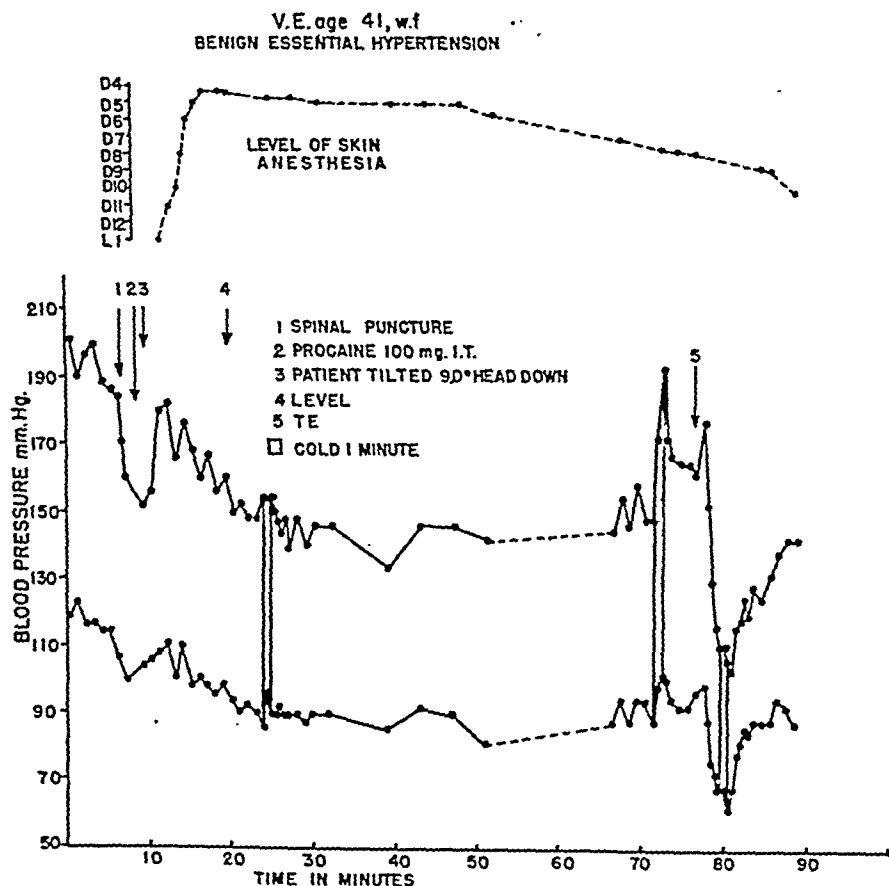


FIG. 5. EXAMPLE ILLUSTRATING THE EFFECT OF VARYING DEGREES OF AUTONOMIC BLOCKADE ON THE COLD PRESSOR RESPONSE

Note that when the level of spinal anesthesia is at D4-5 the cold pressor response is eliminated. At a lower level of D7 the cold pressor response is fully active. After the administration of TEAC (TE) at this level the response is again abolished. This would seem to indicate that in this particular patient clinically significant cold pressor reactivity emanates from the spinal cord between segments D4 and D7.

sponse was abolished by autonomic blockade.⁴ In those instances where the cold minute exposure coincided with the upswing of the TEAC response, it can be seen that the small rises in blood pressure during the cold minute were actually part of the

recovery phase of the blood pressure following the evanescent peak action of the drug. In addition such cold minute patterns differ from those of the cold pressor reaction in that there is no subsequent return of pressure to control pre-test level.

It has been pointed out elsewhere (1) that in hypertensives who demonstrate an active neurogenic component the level of the blood pressure tends to vary directly with the anatomical extent of the arteriolar bed under active vaso motor control. The present results demonstrate that the same concept applies to variations in pressor reactivity in a given individual. It will be noted that sympathetic block (TEAC) abolished the cold pressor reaction. Release of varying portions

⁴ An additional patient studied after the completion of this series, showed a very vigorous control cold pressor reaction which was not affected by spinal anesthesia to T6 or by the injection of 4 cc. (400 mm.) of TEAC. Administration of 8 cc. (800 mm.) of TEAC completely abolished the cold pressor response. This would indicate (as might be expected) that the size of the fully effective dose of TEAC may vary among different patients. The effect on the cold pressor reaction might well be used to gauge the effectiveness of dosage in producing autonomic blockade in individual patients.

of the arteriolar bed from active vasomotor control (spinal anesthesia of varying extent) produces dampening of the response, the degree of which tends to vary with the extent of "denervation." As in the case of the effect on the level of the blood pressure, although the relationship tends to be a direct one, the smallest remaining portion of the arteriolar bed which is capable of exciting clinically significant cold pressor reactivity appears to vary from patient to patient. This probably accounts for the finding that a standard operative procedure (lumbodorsal splanchnicectomy) has produced no uniformity of effect on the pattern of the cold pressor response in a large series of patients (6). The findings in the patient, L. L. (Figure 3), tested after partial sympathectomy, are consistent with this view and suggest that a portion of the sympathetic system large enough to exert clinically significant pressor reactivity was left intact. Although there are not enough determinations in this series to be significant in this respect, the fact that in 6 out of 7 patients, anesthetic levels between T3 and T4 were effective in totally eliminating the response, would suggest that most of the significant cold pressor reactivity is controlled from below this level in the majority of patients.

The demonstration of the neurogenic character of the "cold minute" rise is helpful in leading to an understanding of the delayed cold pressor response seen in exaggerated form in 1 patient (E. H., Figure 4), and to a lesser but significant degree in 11 others. This pressor phenomenon may be humoral in character because it occurs at a time when the sympathetic impulses are blocked at the ganglia, and while the arterioles are still responsive to certain humoral pressor substances. Similarly it also seems likely that the initial rise in blood pressure observed after TEAC in 6 of the patients is humoral in origin.

It is of interest to note that the patient having such a vigorous delayed pressor response exhibited a slow return of his blood pressure to the pre-test baseline during the control cold pressor test. This would suggest that the delayed recovery seen in some patients following the cold pressor test is dependent upon a humoral mechanism in contrast to the neurogenic cold minute phase. In this experiment (Figure 4) the patient exhibited marked anxiety in response to the test situation, and it seems likely that the humoral substance respons-

ible for the response under consideration may have been epinephrine.⁵

Likewise, the fact that the cold pressor response was eliminated in those patients who failed to show a depressor response to the administration of TEAC would suggest that this apparent inefficacy of the drug was due to the maintenance of the blood pressure by a humoral agent, rather than to a failure of the drug to produce autonomic paralysis. This concept is further supported by the findings reported elsewhere (7) that when TEAC is given repeatedly the type of blood pressure response may vary a good deal from day to day so that a patient who fails to react with a large depressor response at one time may do so on a different occasion.

SUMMARY AND CONCLUSIONS

1. Intravenous administration of TEAC to each of 20 patients produced significant lowering of the blood pressure in 14 (70 per cent). Each of the 6 patients (30 per cent) who failed to obtain a depressor response exhibited an initial transient rise in blood pressure following the administration of the drug. It is felt that this rise originates in a humoral pressor mechanism.

2. Following the administration of TEAC the cold pressor response was eliminated in all patients tested, indicating that the mechanism of the cold pressor response is a neurogenic one.

3. The evidence suggests that the cold pressor response may be useful in evaluating procedures designed to eliminate autonomic tone in hypertension.

4. Comparison of the cold pressor responses under varying degrees of spinal anesthesia with those under total autonomic blockade (TEAC) reveals a relationship between the extent of the arteriolar bed under active vasomotor control and the degree of reactivity to the cold pressor test.

5. Delayed cold pressor response, which occurred in 12 out of 20 patients under autonomic

⁵ It may be argued that this is unlikely since the adrenal medulla is innervated by sympathetic fibers whose action should be blocked by the effect of TEAC. Actually the cells of the adrenal medulla are unique in that they are innervated by preganglionic fibers, being themselves analogous to the cells of the sympathetic ganglia. TEAC may not block this connection as it does those at the ganglia. To our knowledge no information on this point is available.

blockade produced by TEAC, can best be explained by a humoral mechanism. It is suggested that the delayed recovery phase of the cold pressor response seen in some patients may be a manifestation of this phenomenon.

6. It is suggested that failure in some cases to obtain depressor responses to TEAC may be due to the presence of a humoral pressor substance rather than to failure of the drug to produce a satisfactory autonomic blockade.

7. The data suggest that both humoral and neurogenic (autonomic) mechanisms may interact as factors in hypertension.

ACKNOWLEDGMENTS

The authors would like to express their appreciation to Miss Mary A. Costello, Chief Anesthetist of the Department of Surgery, University of Cincinnati, and her assistants for their aid in administering the spinal anesthetics. The assistance of Mrs. Jane K. Friedlander and Dr. Joseph E. Levinson is also gratefully acknowledged.

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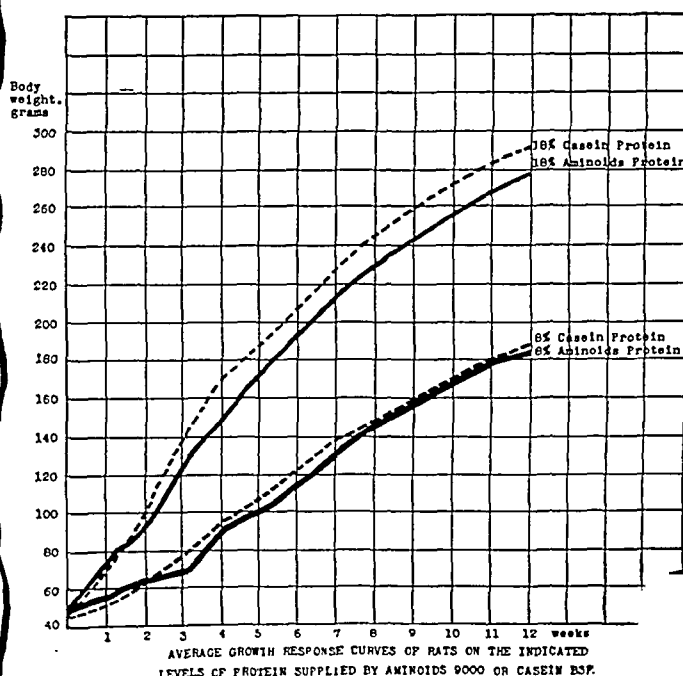
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THE ORAL AND PARENTERAL PHENYLALANINE REQUIREMENTS FOR NITROGEN EQUILIBRIUM IN MAN¹

By RICHARD D. ECKHARDT AND CHARLES S. DAVIDSON
WITH THE TECHNICAL ASSISTANCE OF ELAINE HIRSHBERG

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(Received for publication September 12, 1947)

From published data on the minimum quantity of whole protein (milk, soy flour, white flour, and egg) required (1) or estimated (2) to maintain nitrogen balance in adults, and from analytical data of the amino acid content of these proteins (3), Harte and Travers (4) have calculated the "minimum" requirements for man of the essential amino acids. For phenylalanine, 1.4 Gm. daily was considered to be sufficient. The observations reported here indicate that the daily phenylalanine requirement for man is greater when the daily protein intake (protein hydrolysate) is given intravenously than when given orally. These studies were made during the course of investigations employing as a sole source of nitrogen a 10 per cent solution of amino acids² prepared by complete acid hydrolysis of casein, largely freed of glutamic and aspartic acids, and supplemented with dl-tryptophane, dl-methionine, and glycine (5, 6).

MATERIALS AND METHODS

In Table I the composition of two lots of the solution of amino acids with respect to the 8 amino acids essential for man (7) and for arginine and histidine is shown and is compared to that of casein. The results are based on analyses by microbiological assay (8, 9, 10). Likewise the quantity of amino acids in 1,000 cc. of this 10 per cent solution (average of the two nearly identical lots) is compared with the "minimum" amino acid requirements calculated by Harte and Travers (4). It is apparent that from 2 to 15 times these "minimum" quantities of amino acids are supplied in 1,000 cc. of the solution with the exception of phenylalanine, which might there be expected to be the "limiting" amino acid in this solution. The low phenylalanine content of these particular lots (1.7 per cent and 1.9 per cent) enabled us to investigate the effect on the nitrogen bal-

ance in normal human subjects of this "limiting" essential amino acid. The solution of amino acids employed now regularly contains at least 4 per cent of l-phenylalanine.

A basal diet essentially devoid of protein (supplying 0.1 to 0.5 Gm. nitrogen daily) but adequate in calories, vitamins, and salts was used in all studies. Its composition has been described previously (11). All amino acid analyses were by microbiological assay (8). Since the unnatural isomer of phenylalanine is neither readily utilized by man (12) nor detected by microbiological assay (8), all values presented here are for the naturally occurring l-form. The daily urine and pooled stool nitrogen analyses were determined by the standard micro- or macro-Kjeldahl methods. The alpha amino nitrogen was determined by the gasometric ninhydrin method as described by Hamilton and Van Slyke for plasma (13), and by Van Slyke, MacFadyen and Hamilton for urine (14).

TABLE I

Comparison of the composition of the solution of amino acids with casein, and the quantity of amino acids in 1,000 cc. of the 10 per cent solution with the "minimum" amino acid requirements for man

Amino acid	Amino acid solution* (l-form) Gm. per liter of 10 per cent solution		Casein† Gm. per 100 Gm.			"Mini- mum" amino acid require- ment for man‡	Amino acid solution (Average of lots A and B) 1,000 cc. 10 per cent
	Lot A	Lot B	a	b	c		
Arginine	5.9	5.2	3.9	3.6	3.7	1.2	5.6
Histidine	2.2	1.9	2.8	2.6	3.0	0.5	2.1
Isoleucine	6.0	6.2	5.6	6.4	8.6	1.2	16.1
Leucine ††	12.2	11.5	9.9	9.9	10.5	1.7	11.9
Lysine	13.0	10.8	7.7	8.3	6.7	0.8	11.9
Methionine	5.8	5.4	2.6	2.6	3.1	0.5	5.6
Phenylalanine	1.7	1.9	5.9	5.2	4.8	1.4	1.8
Threonine	1.9	1.9	4.2	4.2	4.6	1.0	1.9
Tryptophane	0.9	0.8	1.1	1.2	1.4	0.4	0.9
Valine	5.5	6.0	6.7	6.2	5.8	1.1	5.8

* Also contains 0.9 Gm. dl-tryptophane, 2.5 Gm. dl-methionine, and 21.6 Gm. glycine per liter of 10 per cent solution. Microbiological assay.

†a—Stokes et al. (8).

b—Hobson and Krueger (9).

c—Pearce et al. (10).

‡Harte and Travers (4).

¹The expenses of this investigation were defrayed in part by a grant from Merck and Company, Inc., to Harvard University.

²Developed and supplied by Merck and Company, Inc., Rahway, New Jersey.

The basal diet was ingested at regular meal hours. The solution of amino acids, when administered intravenously, was injected in 1 to 2 hours once daily without added glucose, and was preceded and followed by food by mouth so that approximately 1,000 calories were given within 1 hour before and 2 hours after the infusion. When the intravenously injected phenylalanine-poor solution of amino acids was supplemented by phenylalanine by mouth, the oral phenylalanine (except in one instance mentioned later) was given approximately 1 to 2 hours prior to the intravenous infusion. The solution of amino acids, when administered orally, was ingested in several divided feedings per day together with the basal protein-free diet.

RESULTS

In Table II are recorded the nitrogen and phenylalanine intake and excretion of 3 normal adult males, each of whom received orally or intravenously 1,000 cc. of the solution of amino acids daily as the sole source of nitrogen. As will be noted (Table II and Figure 1), subject P. C.

was in nitrogen equilibrium during an initial period while receiving, in addition to the solution of amino acids intravenously, an orally administered supplement of phenylalanine. Together, these supplied a total of from 2.7 to 2.9 Gm. of phenylalanine daily. When oral phenylalanine supplementation was omitted in the second period, reducing the intake of this amino acid to 1.9 Gm., a negative nitrogen balance promptly resulted. However, subsequent administration during a third period of the amino acid solution orally in several feedings daily for 8 days without the phenylalanine supplement (phenylalanine intake 1.9 Gm. daily) resulted in nitrogen equilibrium except for days 8 and 12. Subjects E. B. and M. D. O. (Table II), who were both given the amino acid solution by rapid intravenous infusion once daily, likewise substantiate the finding that 1.9 Gm. of phenylalanine when given by the intravenous route did not maintain nitrogen equilib-

TABLE II

Nitrogen and phenylalanine intake and excretion in 3 subjects given 1,000 cc. of the 10 per cent solution of amino acids daily

Subject	Period	Day	Nitrogen total daily intake grams per day		Nitrogen total daily output grams per day		Nitrogen balance	l-Phenylalanine total daily intake grams per day		l-Phenyl- alanine total daily output	Adminis- tered l-phenyl- alanine excreted in urine
			i.v.	Oral	Urine	Stool		i.v.	Oral		
P. C.	I	1	15.7	0.5	13.4	1.0	+1.8	1.7	1.0	57.7	2.1
		2	15.7	0.5	12.1	1.0	+3.1	1.7	1.0		
		3	14.3	0.5	14.6	1.0	-0.8	1.9	1.0		
		4	14.3	0.5	13.7	1.0	+0.1	1.9	1.0		
		5	14.3	0.5	13.6	1.0	+0.2	1.9	1.0		
	II	6	14.3	0.4	15.3	1.0	-1.6	1.9	0	218.0	11.5
		7	14.3	0.4	15.7	1.0	-2.0	1.9	0		
	III	8	0	14.7	17.5	0.9	-3.6	0	1.9	32.9	1.7
		9	0	14.7	13.5	0.9	+0.3	0	1.9		
		10	0	14.7	13.6	0.9	+0.2	0	1.9		
		11	0	14.7	13.7	0.9	+0.1	0	1.9		
		12	0	14.7	15.2	0.9	-1.4	0	1.9	32.7	1.7
		13	0	14.7	13.6	0.9	+0.2	0	1.9		
		14	0	14.7	13.4	0.9	+0.4	0	1.9		
		15	0	14.7	13.1	0.9	+0.7	0	1.9		
E. B.	I	1	15.7	0.5	15.4	0.5	+0.3	1.7	1.0	130.0	4.8
		2	15.7	0.5	15.9	0.5	-0.2	1.7	1.0		
M. D. O.	I	1	13.7	0.1	14.5	0.2	-0.9	1.8	0	200.0	10.5
		2	14.3	0.1	12.5	0.2	+1.7	1.9	0		
		3	14.3	0.1	15.9	0.2	-1.7	1.9	0		
		4	14.3	0.1	15.0	0.2	-0.8	1.9	0	221.0	11.6
		5	14.3	0.1	16.6	0.2	-2.4	1.9	0		
		6	14.3	0.1	15.2	0.2	-1.0	1.9	0		
		7	14.3	0.1	15.4	0.2	-1.2	1.9	0		

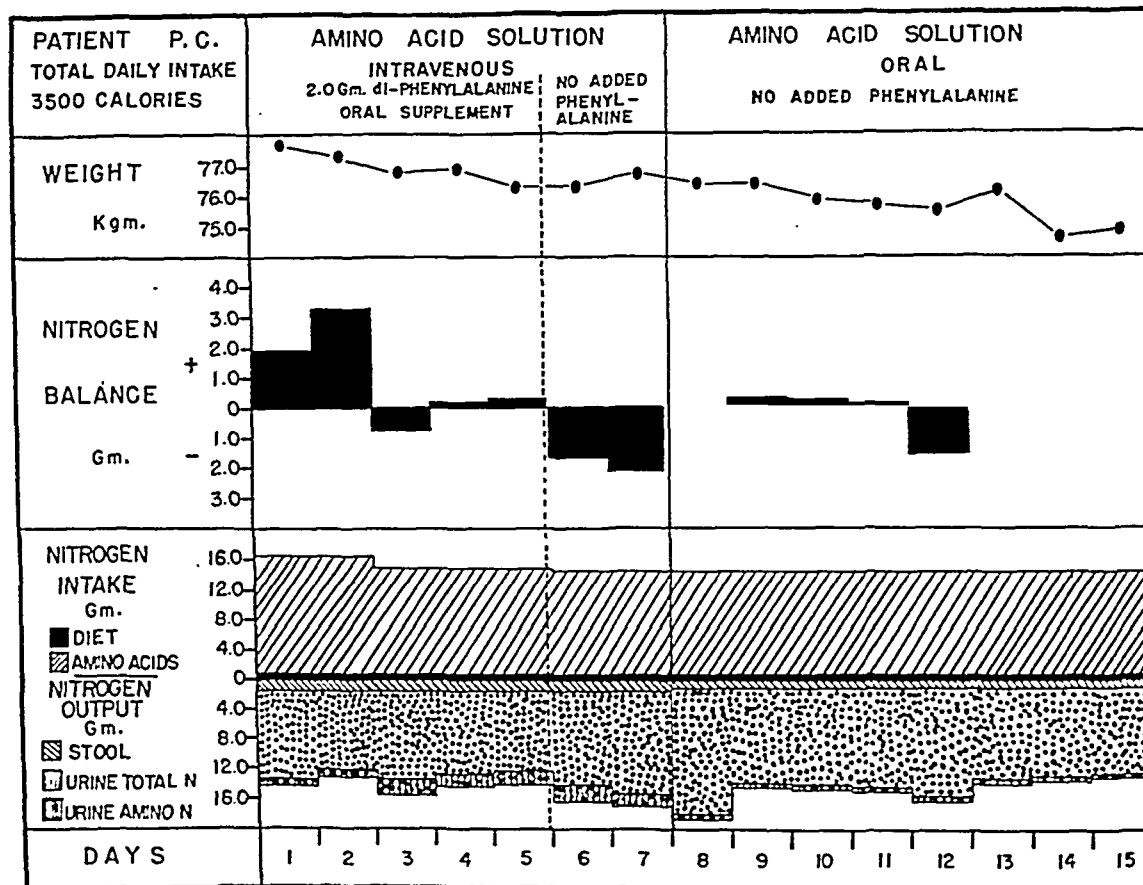


FIG. 1. NITROGEN BALANCE OF PATIENT P. C. WHILE RECEIVING AMINO ACID SOLUTION INTRAVENOUSLY, THEN ORALLY

rium until additional phenylalanine was provided.

Subjects P. C. and E. B. who were in nitrogen equilibrium while receiving the parenteral amino acid solution supplemented with phenylalanine given by mouth excreted 58 mgm. and 189 mgm. (P. C.) and 130 mgm. (E. B.) of microbiologically available free phenylalanine in the urine daily. These amounts represent 2.1 per cent, 6.5 per cent, and 4.8 per cent, respectively, of the administered l-phenylalanine. Subjects P. C. and M. D. O., while receiving the intravenous amino acid solution without additional phenylalanine, were in negative nitrogen balance, P. C. excreting 218 mgm., and M. D. O. excreting 200 mgm. and 221 mgm. of free urinary phenylalanine. This represented an increase to 11.5 per cent, 10.5 per cent, and 11.6 per cent, respectively, of that administered. While in nitrogen equilibrium on the unsupplemented low-phenylalanine amino acid

solution administered orally, subject P. C. excreted only 33 mgm. of phenylalanine daily, or 1.7 per cent of the dosage fed.

DISCUSSION

In this laboratory the average phenylalanine excretion in normal adult males on an *ad libitum* feeding is 14 mgm. daily (range 7 to 23 mgm. for 8 individuals [15]). One individual who maintained weight and nitrogen balance on 89 Gm. of whole ("Labco") casein daily as the sole source of nitrogen excreted 0.5 per cent of the ingested phenylalanine (5.0 Gm. fed and 24 mgm. excreted daily [15]). Others (16 to 18) also have found but minute quantities (3 to 10 mgm.) excreted daily by normal individuals. In contrast is the somewhat greater absolute and percentage excretion of phenylalanine when the solution of amino acids was ingested orally by subject P. C.

TABLE III

Urine alpha amino nitrogen excretion in normal subjects given whole casein orally, the amino acid solution orally, and the amino acid solution intravenously

Subject	Protein source	Route of administration	Alpha amino nitrogen administered	Alpha amino nitrogen excreted in urine mgm. per 4-hour period			
				Control fasting	After administration	Net urinary loss	
			mgm.			mgm.	per cent
R. D. E.	Whole casein	Oral	5,440	28.7	52.1	23.4	0.4
C. S. D.	Whole casein	Oral	5,440	26.4	43.3	16.9	0.3
R. D. E.	Amino acid solution	Oral	5,440	28.7	181.3	152.6	2.8
C. S. D.	Amino acid solution	Oral	5,440	26.4	189.2	162.8	3.0
8 Normal adult males (average)	Amino acid solution	Intravenous	6,000	17.2	581.5	564.3	9.4

Since West, Wilson, and Eyles (19) found higher blood levels of amino nitrogen in infants who had ingested an acid hydrolysate of casein than after ingestion of an equivalent amount of whole protein (casein), we felt that a higher blood level

might account for the increased excretion of phenylalanine in subject P. C. Two of the present authors (R. D. E. and C. S. D.), fasting on two successive days, ingested equivalent amounts of nitrogen first as whole casein and on the second day as the amino acid solution. The blood amino nitrogen values obtained hourly post-ingestion were variable for both subjects and were not markedly different after the ingestion of either protein, but both subjects excreted several times greater quantities of amino nitrogen in the urine (4 hours post-ingestion) after the amino acid ingestion than after the casein feeding. In Table III and Figure 2 are presented data on the absolute and percentage excretion of amino nitrogen following the administration of equivalent amounts of nitrogen as whole casein orally, as the amino acid solution orally, and as the amino acid solution intravenously (20). The average net urinary amino nitrogen loss during the first 4 hours³ after the orally administered whole casein was 20.1 mgm. (0.4 per cent), after the orally ingested amino acid

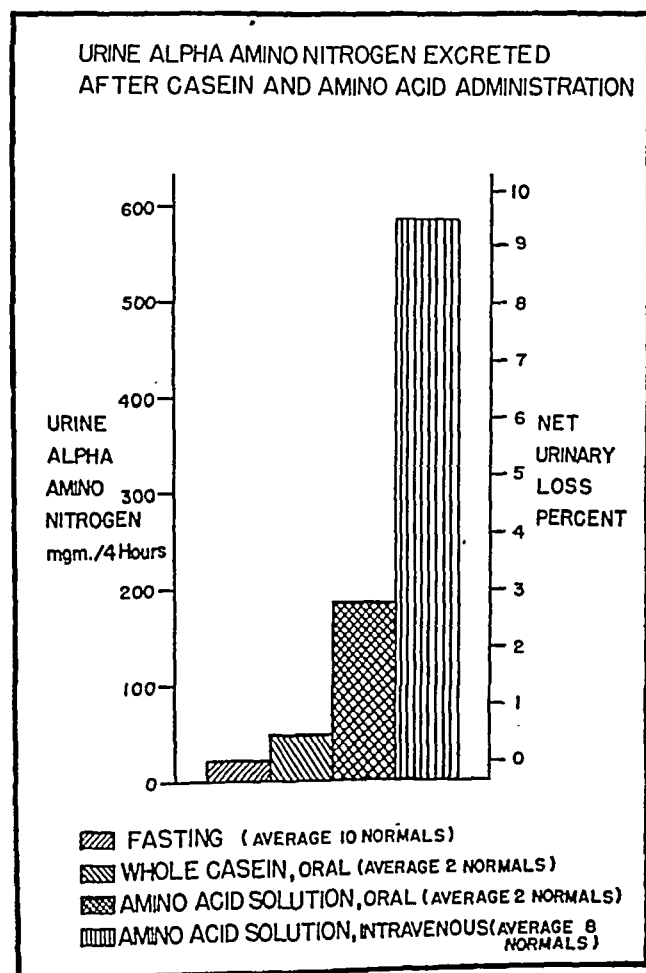


FIG. 2

³ A 4-hour period of urine collections was chosen since the loss of amino nitrogen in the urine due to the infusion of the solution of amino acids was complete within this period, by which time the elevated blood amino nitrogen value had returned to normal (20). However, since the elevated blood amino acid values following whole protein and amino acids by mouth do not return entirely to normal by this time (19), apparently reflecting digestion and absorption, a longer collection period would have been more desirable. Nevertheless, it is our experience that the subsequent loss of amino nitrogen is so small it would not detract from the significance of these observations.

solution was 157.7 mgm. (2.9 per cent), and after the intravenously injected amino acid solution was 564.3 mgm. (9.4 per cent). Thus, although the finding in subject P. C. of increased urinary phenylalanine excretion when the amino acid solution rather than whole protein was given by mouth could not be definitely related to high blood amino nitrogen values, it was associated with an increased excretion of amino nitrogen, including all the amino acids fed, and was not due to the selective excretion of phenylalanine.

When the solution of amino acids was administered parenterally to the subjects reported here, they excreted 4 to 16 times the quantity of phenylalanine normally found in the urine, presumably because of the marked rise in blood amino acid values following the rapid intravenous infusion of the amino acid solution. The absolute quantity of phenylalanine excreted after parenteral infusion was actually slightly less when 2.0 Gm. of dl-phenylalanine was added as an oral supplement than when none was given. Furthermore the percentage excretion of the phenylalanine administered was from 2 to 5 times greater with no supplementation than when supplemental phenylalanine was given orally. This would suggest that at least part of the supplemental phenylalanine was conserved or retained when sufficient was administered to allow nitrogen equilibrium, while when less than the minimum required for nitrogen equilibrium was available, a portion of that given was wasted. Similar observations were made by Pearce, Sauberlich and Baumann (10) who found that mice fed incomplete proteins excreted a much greater percentage of the ingested amino acids in the urine in microbiologically available form than did mice fed complete proteins.

When a protein hydrolysate or mixture of amino acids deficient in one or more essential amino acids is given in order to maintain nitrogen equilibrium, the limiting amino acid(s) must be so administered that all the essential amino acids are present in the body at approximately the same time (21). Recent studies have suggested that the maximum time interval may be as short as one hour (22). The slight negative nitrogen balance of subject P. C. on day 3 may be interpreted in this way, for he received the oral phenylalanine supplement one-half hour after the infusion had been administered; or, of course, it may merely be

a chance observation. At other times the supplemental phenylalanine was administered orally 1 to 2 hours prior to the infusion, for maximum blood levels (measured microbiologically) have been shown to occur after such an interval following ingestion (23). A more ideal procedure would have been to administer this amino acid parenterally with the phenylalanine-deficient solution of amino acids. The intravenous injections of the amino acid solution were preceded and followed by approximately 1,000 food calories by mouth in order to achieve maximum retention of the nitrogen (24).

The smaller quantity of phenylalanine required daily for maintenance of nitrogen balance when the solution of amino acids was given orally rather than intravenously may have been due to more efficient and economical utilization with divided oral feedings than with the single rapid daily intravenous injection. Further, the greatly increased urinary excretion of free phenylalanine and other amino acids following the intravenous infusions as compared to that following the oral feedings must have decreased the quantity of free phenylalanine available for metabolic purposes. That 1.9 Gm. of phenylalanine daily does not necessarily represent the "minimum" oral requirement is acknowledged. The smaller calculated value of Harte and Travers (4) of 1.4 Gm. daily may be nearer the true minimum. Likewise, 2.7 Gm. of phenylalanine may be greater than the minimum required for parenteral administration, but certainly under the conditions of our observations, 1.9 Gm. daily did not suffice. It must be borne in mind that man can probably partially utilize the unnatural isomer of phenylalanine (12). Thus, when we gave 2.0 Gm. of dl-phenylalanine as an oral supplement, more than 1.0 Gm. (the l-form) may have been nutritionally available. Minimum oral requirements for amino acids will undoubtedly be found insufficient when the conditions of the study are altered. Thus the quantity required may be expected to be greater when hydrolyzed rather than when whole protein is administered orally, and still greater when parenteral alimentation is used.

CONCLUSIONS

1. A solution of amino acids containing 1.9 Gm. of l-phenylalanine will maintain nitrogen balance

in normal adult man if given orally in several divided feedings daily.

2. The same solution of amino acids is not capable of maintaining nitrogen balance if given parenterally in one rapid injection daily unless additional phenylalanine is simultaneously provided. Under these conditions, 2.7 Gm. of l-phenylalanine will maintain nitrogen balance.

3. The urinary loss of phenylalanine was greater both in absolute and in percentage amounts of that administered when less than the minimum requirement of the amino acid was given and negative nitrogen balance resulted, than when sufficient of the amino acid was available for nitrogen equilibrium.

4. The oral administration of the amino acid solution resulted in an 8 times greater urinary loss of amino acids than when whole protein was fed. The parenteral administration of the amino acid solution resulted in a 28-fold increase.

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DEPRESSION OF THE EXOGENOUS CREATININE/INULIN OR THIOSULFATE CLEARANCE RATIOS IN MAN BY DIODRAST AND p-AMINOHIPPURIC ACID¹

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Exogenous creatinine is excreted by the renal tubules of the aglomerular fish (1 to 4), the glomerular dogfish (5, 6), teleost (7) and chicken (8), but not by the frog (9), turtle (10), dog (11 to 14), sheep (15), seal (16), rabbit (17) or cat (18). Among the mammals tubular excretion is recorded only in the anthropoid apes (19, 20) and man (21).

The evidence for tubular excretion in man, as initially advanced by Shannon, consists of the facts that (1) the exogenous creatinine/inulin clearance ratio exceeds 1.0 (ranging from 1.2 to 1.7) (21, 22, 23); (2) this ratio is depressed towards 1.0 by raising the plasma level of creatinine (21); (3) at all plasma levels, the creatinine/inulin clearance ratio is depressed towards 1.0 by phlorhizin (21) which is known to depress the tubular excretion of phenol red (24) and diodrast (25).

Winkler and Parra (26) observed that after the ingestion of creatinine, both the creatinine clearance and the creatinine/sucrose clearance ratio behaved erratically and generally fell as the experiment proceeded, and they believed that this progressive fall with time represented the same phenomenon as the self-depression of the clearance at high plasma levels. Shannon and Ranges (27) have, however, presented evidence to refute this view. They have shown that (a) the creatinine/inulin clearance ratio does not fall markedly with time if the plasma level is maintained by the continuous infusion of creatinine, (b) a second dose of creatinine elevates the clearance ratio, after this has been depressed by the lapse of time, towards the values observed shortly after a first dose, and (c) this elevation occurs only if the ratio has been depressed by virtue of the prolonged circulation of creatinine in the body. These ob-

servations appear to exclude 'fatigue' and 'stimulation' of the excretory mechanism. They accord with the possibility suggested by the above investigators; namely, that in the body creatinine is converted in part to a compound which, though still giving the Jaffe reaction, is less readily excreted by the tubules; or, alternatively, to a compound which blocks excretion by virtue of a high competitive affinity for some component of the excretion mechanism. This latter possibility is rendered more plausible by the recent description of caronamide (28), a compound not excreted by the tubules but one which blocks the excretion of some other substances (phenol red, diodrast, penicillin, etc.).

The evidence for the tubular excretion of exogenous creatinine in man has not been convincing to all writers (29) and it has seemed desirable to examine the matter further.

Substances excreted by the renal tubules appear in general to interfere in the excretion of other substances, presumably by competition for some enzyme system or source of energy. Thus diodrast and hippuran depress the excretion of phenol red and *vice-versa* (30, 31), hippuric acid depresses the excretion of phenol red (32), as do iopax, skiodan and neoiopax (33), while diodrast (34) and p-aminohippuric acid (35) depress the tubular excretion of penicillin.²

EXPERIMENTAL

It seemed possible that if creatinine is excreted by tubules in man, the simultaneous excretion of large quantities of diodrast or p-aminohippuric acid (PAH) would depress the creatinine/inulin clearance ratio. Consequently, we have made a number of observations in which

²A possible exception to this interference phenomenon is observed in the dogfish, where creatinine, though copiously excreted by the renal tubules, has only slight effect on the simultaneous excretion of phenol red. The single experiment reported is, however, equivocal (21).

¹Aided by a grant from the Commonwealth Fund.

this ratio was first determined during three consecutive control periods of some 10 minutes each. They were examined again during a second series of three consecutive periods 20 to 60 minutes after the intravenous administration of large doses of diodrast (15 to 30 cc. of 35 per cent solution) or PAH (30 to 50 cc. of 20 per cent solution), supported by the intravenous infusion of sufficient quantities of these substances to maintain high plasma concentrations. (See Table I for plasma concentrations.) Creatinine was administered intravenously in normal saline in priming and sustaining infusions in sufficient doses to maintain a plasma level of 7 to 15 mg. per cent. Since Newman, Gilman and Phillips (36) have shown that the thiosulfate clearance is essentially identical with the inulin clearance in man,³ we have included a number of creatinine/thiosulfate clearance comparisons carried out in the same manner.

³ This identity has been amply confirmed in this laboratory in observations not reported here.

Clearances, performed by the standard infusion technic with priming and sustaining infusions and bladder catheterization (37), were measured in convalescent female patients without renal disease selected from various wards of the Third (New York University) Division of Bellevue Hospital. A 1:10 or 1:15 cadmium sulfate filtrate of plasma (32) was used for creatinine and inulin determination, and a 1:10 tungstate filtrate (36) for thiosulfate. Creatinine was determined by the alkaline picrate method (32), inulin determined by Harrison's method (38) and thiosulfate by the method of Newman, Gilman and Phillips (36).

RESULTS

Our results are shown in Table I, which presents all the data pertinent to the interpretation of the experiments.

During the control periods we have consistently observed an inulin/creatinine clearance ratio in

TABLE I

The effects of large doses of p-aminohippuric acid and diodrast on the creatinine/inulin and creatinine/thiosulfate clearance ratios in man

Date 1946	Patient	Plasma concentration*				Clearance			Clearance ratios			
		Creat.	Inulin	Thio.	PAH	Creat.	Inulin	Thio.	Creat. Inulin	Creat. Thio.	Thio. Inulin	
		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		cc. per min.	cc. per min.	cc. per min.				
10/9	DP	11.7-10.9		19.1-19.7		177		143		1.23		Control
		10.9-11.4		19.0-19.4		125		131		0.956		After diodrast
10/11	JL	20.4-14.8		25.5-23.7		135		119		1.14		Control
		14.8-13.9		22.5-22.0		101		103		0.974		After diodrast
10/14	DP	10.8-11.3		20.8-21.5		149		101		1.48		Control
		11.3-11.8		20.5-19.6		115		94		1.23		After diodrast
11/15	CL	9.6-7.4	31.0-16.2	18.3-12.6		220	174	168	1.29	1.31	0.974	Control
		8.2-8.2	17.7-17.0	13.7-12.5		145	133	146	1.09	0.988	1.10	After diodrast
								Avg.		1.29		Control
								Avg.		1.04		After diodrast
10/16	AP	10.8-9.7		18.2-15.2		180		151		1.20		Control
		8.4-8.2		11.7-10.8	73-92	113		116		0.984		After PAH
10/21	DP	12.4-11.9		20.4-20.4		181		150		1.21		Control
		10.8-9.9		16.4-16.4	57-43	141		134		1.05		After PAH
10/23	VA	9.2-9.2	16.0-14.2	17.0-17.9		164	139	137	1.20	1.21	0.988	Control
		8.8-8.8	10.7-11.2	16.0-15.9	42-57	127	134	125	0.948	1.02	0.929	After PAH
10/30	DK	9.1-8.0	22.0-17.2	15.0-12.9		229	182	175	1.26	1.31	0.967	Control
		6.2-6.6	11.9-11.8	11.4-10.0	17-29	181	149	154	1.22	1.19	1.03	After PAH
11/1	DK	10.3-9.7	20.0-15.0	18.5-17.3		231	181	164	1.28	1.42	0.904	Control
		9.7-9.1	12.8-11.3	16.7-15.4	58-65	168	141	138	1.19	1.23	0.969	After PAH
11/6	AA	12.8-12.2	25.6-21.2	24.3-22.8		158	129	124	1.23	1.27	0.966	Control
		11.4-11.4	17.8-17.6	20.7-21.2	60-65	125	116	121	1.08	1.03	1.05	After PAH
11/8	CM	14.3-12.8	22.2-20.0	20.6-19.8		139	109	110	1.28	1.27	1.00	Control
		11.4-11.6	16.3-17.8	19.8-19.1	40-51	98.9	94.6	83.9	1.05	1.18	0.891	After PAH
11/13	JJ	7.6-7.6	15.0-13.8	14.7-12.8		215	172	174	1.24	1.14	0.983	Control
		9.0-8.8	16.7-14.4	10.7-9.5	35-38	166	133	158	(1.25)†	1.05	(1.19)†	After PAH
								Avg.	1.25	1.25	0.969	Control
								Avg.	1.10	1.09	0.990	After PAH

* Plasma concentrations of diodrast not determined.

† Omitted from averages.

excess of 1.0 (range 1.12 to 1.29) and a creatinine/thiosulfate clearance ratio in excess of 1.0 (1.14 to 1.48).

After the administration of diodrast or PAH these ratios (with the single exception of the creatinine/inulin ratio in J. J.) have dropped, in many cases to values slightly under or slightly above 1.0. In a single subject, diodrast lowered the creatinine/inulin clearance ratio from 1.29 to 1.09. In 6 subjects this ratio averaged 1.25 before, and 1.10 after PAH. The creatinine/thiosulfate ratio in 4 subjects averaged 1.29 before and 1.04 after diodrast; while in 8 subjects this ratio averaged 1.25 before and 1.09 after PAH. (The last figure omits J. J. where an aberrantly large thiosulfate/inulin ratio of 1.19 indicates a possible erroneously low inulin clearance in the last half of the experiment.)

Our data on the simultaneous thiosulfate/inulin clearance ratio (average of 21 periods in 7 subjects, 0.97, range 0.891 to 1.10, excluding J. J.) confirm Newman, Gilman and Phillips (36) in their conclusion that the thiosulfate and inulin clearances are identical in man.* After the administration of diodrast or PAH this ratio averaged 0.99 (18 periods in 6 subjects), indicating that these substances do not disturb the excretion of either thiosulfate or inulin.

If it is argued that the exogenous creatinine clearance is at the level of glomerular filtration in man, then a creatinine/inulin ratio in excess of 1.0 indicates active tubular reabsorption of inulin, since inulin is completely ultrafiltrable from plasma (39). Reduction of this ratio to or towards 1.0 by the administration of diodrast or PAH could only be explained by supposing (a) that this tubular reabsorption is blocked by these compounds, or (b) that the permeability of the tubules was so increased as to permit creatinine (and to a lesser extent) inulin to escape from the urine to such an extent as approximately to equalize the clearances. Under supposition (a), the fact that the inulin and thiosulfate clearances are, within experimental error, identical before and after the administration of diodrast and PAH would require not only that

thiosulfate be reabsorbed to the same extent as inulin, but that diodrast and PAH have a similar effect in blocking the reabsorption of both compounds, while the failure of diodrast to produce glycosuria would require that this compound block the reabsorption of inulin but not that of glucose. All three possibilities seem to us to be highly improbable. In respect to (b), it will be noted that in all cases the absolute creatinine, inulin and thiosulfate clearances decreased after the administration of large doses of diodrast or PAH, a phenomenon which in respect to the inulin clearance is well recognized (30). This decreased clearance might be interpreted as indicating tubular injury and back-diffusion except for the fact that the clearances tend to return to control values despite the maintenance of high plasma levels of these compounds. It is well known that both compounds when given in large amounts may produce vasomotor and other autonomic responses (burning of the skin, sweating, headache, cramps, nausea and sometimes rectal contraction [40]). The variable and transient depression of renal clearances may reasonably be attributed to disturbances of the glomerular circulation. There is no evidence that substances (diodrast and PAH) copiously excreted by the renal tubules increase their permeability, or that this increase would be of such a nature as to maintain the identity of the inulin and thiosulfate clearances and approximately to equalize these clearances with that of creatinine.

We are therefore inclined to accept the simpler interpretation; namely, that the inulin and thiosulfate clearances are at the level of glomerular filtration and that the exogenous creatinine clearance exceeds both the other clearances because of the tubular excretion of this compound, this tubular excretion being depressed and the creatinine clearance being reduced to or towards the inulin and thiosulfate clearance by diodrast and PAH in consequence of some type of intracellular competition.

This conclusion extends the evidence for the tubular excretion of creatinine as advanced by Shannon (21) and Shannon and Kasper (27), and confirms that reviewed elsewhere on the mechanism of excretion of inulin (32).

*We are inclined to believe that the large scatter in our ratios is in part attributable to variations in the large thiosulfate blank in the plasma, a definite disadvantage in this method as compared with inulin.

SUMMARY

It is confirmed that in normal man the exogenous creatinine clearance exceeds the simultaneous inulin or thiosulfate clearance to an extent clearly beyond the limits of experimental error.

In 39 periods in 7 subjects, the thiosulfate/inulin clearance ratio averages 0.98, confirming the conclusion of Newman, Gilman and Phillips that the thiosulfate clearance is at the level of glomerular filtration.

The administration of large doses of diodrast or PAH (substances known to be copiously excreted by the renal tubules in man) depresses the creatinine/inulin and creatinine/thiosulfate ratio to or towards 1.0. These facts are presented as new evidence for the tubular excretion of exogenous creatinine, and confirm the belief that in man as in other mammals the inulin clearance is at the level of glomerular filtration.

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ON THE BLOOD LACTIC ACID RESPONSE TO MEASURED EXERCISE IN HYPOXIC HUMAN SUBJECTS¹

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When working mammalian muscle tissue is deprived of sufficient oxygen to support oxidative metabolism at a specified work load, lactic acid accumulates in the muscle cells, diffuses into the extracellular fluid and thence into the circulating blood. That the concentration of lactate in the circulating blood is a true indication of tissue lactate concentration in working muscle has been shown by Newman (1). Thus, it is theoretically possible to test the adequacy of a continuing supply of oxygen to working muscle by measuring changes that occur in blood lactate concentration following measured bouts of exercise.

According to the hypothesis of Bang (2), who made extensive studies of the blood lactate response to exercise at sea level barometric pressure, all of the lactic acid formation by muscle during the transition from the resting to the working state occurs before the circulatory and respiratory responses of the organism have collaborated in delivering oxygen to the muscle at the rate required by its work load. When the intricate pattern of reflexes brought into play by the exercise have resulted in hyperpnea, increased cardiac output and local vasodilation in the working muscle, and when oxygen is delivered at a sufficiently rapid rate to support contraction aerobically, lactic acid production practically ceases. Thus, in a subject exercising at a constant rate, Bang (2) found identical blood lactate curves when the work was continued for 10, 15, or 30 minutes.

During the performance of muscular work the organism must make circulatory and respiratory

adjustments which are qualitatively similar to those made during adaptation to hypoxia. A combination of these stresses, therefore, makes possible the detection of comparatively mild degrees of hypoxia. Edwards (3), in Peru, had demonstrated that non-acclimatized subjects showed unusually high blood lactate levels following the performance of measured work at altitudes of about 9,000 feet and above. Broussilovsky (4), in Kiev, reported similar findings in subjects exposed to simulated altitudes of about 9,000 feet and above in a decompression chamber.

The following report is divided into two sections: the first includes a description of the chemical changes in the blood produced by exercise at low barometric pressure, together with an inquiry into the effect of antecedent muscular training on the response; the second is an account of certain experiments designed to elucidate the mechanism of the response.

METHODS

Preparation of subjects. All experiments, including sea level control runs, were done in the decompression chamber at $70^{\circ} \pm 2^{\circ}$ F. The subjects sat at rest for at least one hour before the first blood sample was taken; experiments were begun between two and one-half and four hours after the preceding meal, usually breakfast. When repeated studies were made on the same individual an effort was made to duplicate experimental conditions, especially with respect to composition and time of the last meal, length of rest period, time of day, and activity involved in coming to the laboratory.

Visible sweating was largely avoided by having the subjects wear athletic shorts or playsuits, and by providing an adequate amount of ventilation at all times.

Work load. In the preliminary experiments standing-running to a metronome was first used as the standard exercise. However, it was soon found that this is an unreliable method of assigning reproducible exercise loads. Therefore, the subjects were instructed to do deep knee-bends in time to a metronome at the rate of 30 per minute. On six occasions this amount of exercise proved to have distressing after-effects; five of the subjects complained

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of extreme stiffness of the lower extremities and weakness of the knees, persisting, in some cases, for as long as eight days. One of the subjects was hospitalized two days after he exercised at 15,000 feet when it was found that he had moderately severe "hemoglobinuria." He made a satisfactory recovery, however, in about three days.

In the second series of experiments measured amounts of work were performed on an eddy-current brake cycle ergometer, kindly loaned by Professor E. C. Schneider of Wesleyan University. The instrument was calibrated in kilogram-meters of work per minute at a pedal speed of 60 pedal revolutions per minute, the subject pedalling in time to a metronome mounted between the handle-bars. Almost all of the subjects were able to check themselves within two or three pedal revolutions for three-minute exercise periods after very little training. They were instructed to use their upper extremities as little as possible during the performance of work.

Blood studies. Heparinized arm-vein blood was used in all experiments. In the ergometer experiments an electrically heated gauntlet was placed on the arm about five minutes before each blood sample was to be drawn. While not as effective as immersing the hand in hot water, this procedure causes a vasodilation of the vessels of the hand, increased blood flow, and diminished arteriovenous differences. Incidentally, it also facilitates the performance of ten venepunctures in two hours in subjects whose veins are not prominent.

Protein precipitation was carried out in the chamber. Trichloroacetic acid filtrates for lactate and pyruvate estimations were made within two minutes after the blood was drawn. Lactate was estimated by means of the Evelyn photoelectric colorimeter according to the method of Barker and Summerson (5), and pyruvate, according to the Bueding and Wortis (6) modification of the method of Lu. The glucose method was that of Somogyi (Peters and Van Slyke [7]), and amino acid nitrogen analyses were performed by the technique of Frame, Russell and Wilhelmi (8). Arterial oxygen saturation was estimated by the oximeter method of Millikan (9).

I. A DESCRIPTION OF THE CHEMICAL CHANGES IN THE BLOOD FOLLOWING EXERCISE AT LOW BAROMETRIC PRESSURE

A. Preliminary experiments

The initial experimental design required each subject to perform two short bouts of exercise (deep knee-bends) separated by a 72-minute period of sitting at rest. Blood samples were taken just before, and 5, 15, 30 and 45 minutes after the beginning of each bout. In seven of the nine subjects who did two successive bursts of exercise at sea level, the second burst resulted in a smaller lactate rise than the first. In the other two, the curves were the same.

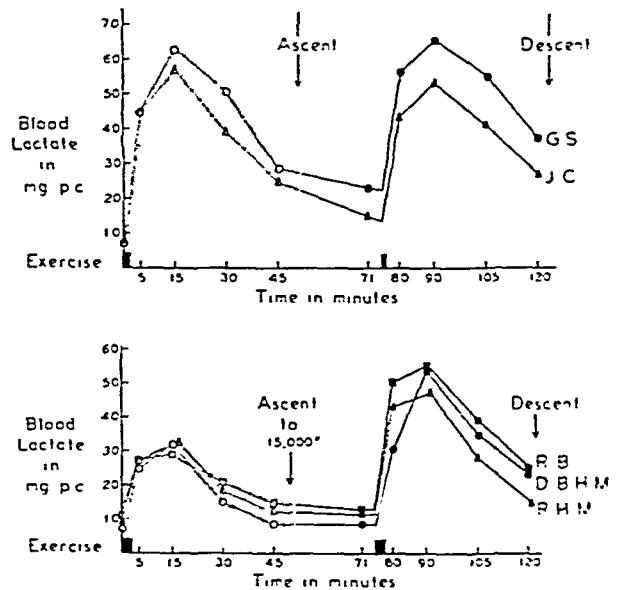


FIG. 1. EFFECT OF EXERCISE AT SEA LEVEL FOLLOWED BY EXERCISE AT 15,000 FEET IN TWO POORLY TRAINED SUBJECTS (G. S. AND J. C.) AND THREE WELL-TRAINED SUBJECTS (R. B., D. B. H. M. AND R. H. M.)

Two young men, both in a poor state of athletic training, exercised to capacity for two minutes at sea level, ascended to 15,000 feet about 45 minutes later, and began to exercise at altitude 75 minutes after the beginning of the first exercise period. The results of these experiments are plotted in Figure 1. Although the duration of exercise in each case was cut from two minutes to one minute and 20 seconds, the configurations of the blood lactate curves at altitude closely resembled those of the sea level curves.

Typical responses of three well-trained athletes are shown in Figure 1. Similar results were obtained in a total of nine experiments on seven subjects. It is apparent that the blood lactate following exercise at 15,000 feet is much higher than it is following the same amount of exercise at sea level. However, since, as has already been stated, the second of two serial bouts of exercise often results in a smaller lactate rise than the first, this experiment does not afford a satisfactory method of comparing performance at altitude and at sea level.

B. Ergometer experiments

Six subjects volunteered to serve for a series of three or four experiments. Two of the men

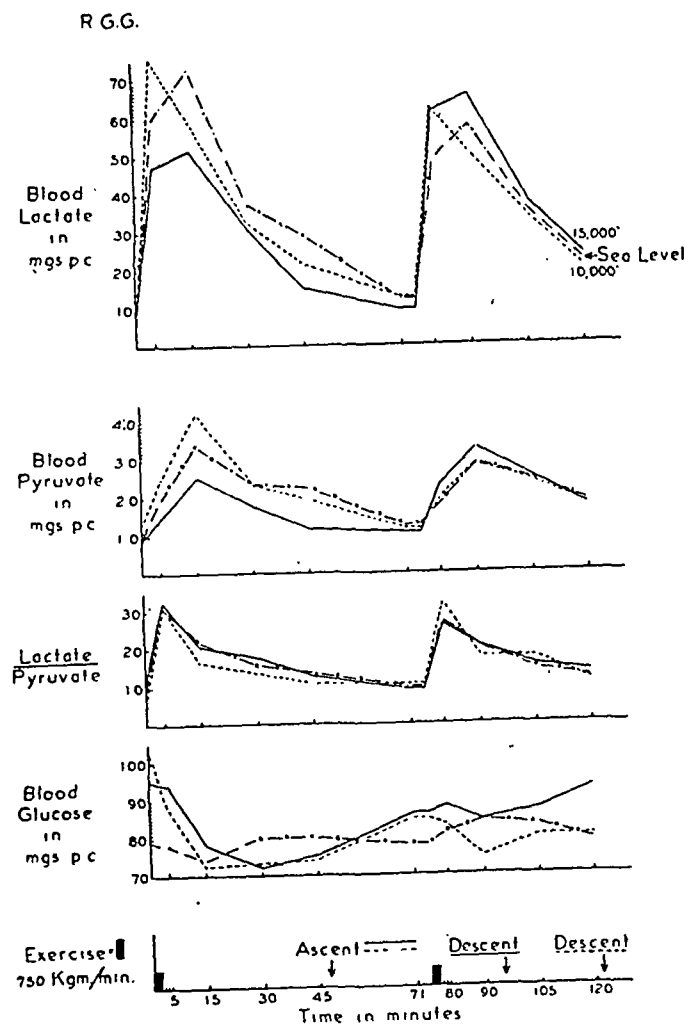


FIG. 2. SUBJECT R. G. G. POORLY TRAINED MALE, AGE 27, HT. 5'10", WT. 170 LBS. EXERCISE: CYCLE ERGOMETER

Both exercise bouts at sea level, 8/12/43. Second exercise bout at 10,000 feet, 9/2/43. Second exercise bout at 15,000 feet, 9/22/43.

were in poor athletic training and had not ridden a bicycle since early boyhood. The other four were well-trained cyclists who often rode from 5 to 15 miles per day. Two of these were arbitrarily assigned heavy work loads, whereas the other two were given moderate ones. The general experimental plan was similar to that used in the preliminary experiments, with the exception that all comparisons of performance at different simulated altitudes were done on the second of the two exercise periods. Thus, each subject exercised once at sea level before doing the critical part of the experiment for each day. This procedure was adopted because it was believed that the second of two consecutive tests would be more likely to

produce uniform responses than the first. In retrospect, the blood lactate responses of the four trained subjects showed rather remarkable internal consistency of performance from one test day to another, even after the first of the two exercise periods.

In addition to lactate and glucose, the pyruvate and amino acid nitrogen content of whole blood were estimated, the volume of packed red blood cells was measured, and the oxygen saturation of the arterial blood was estimated by means of the Millikan oximeter. Pulse rates were counted before and after each exercise bout and after recovery. The results of these experiments are presented graphically in Figures 2 to 6. Since the

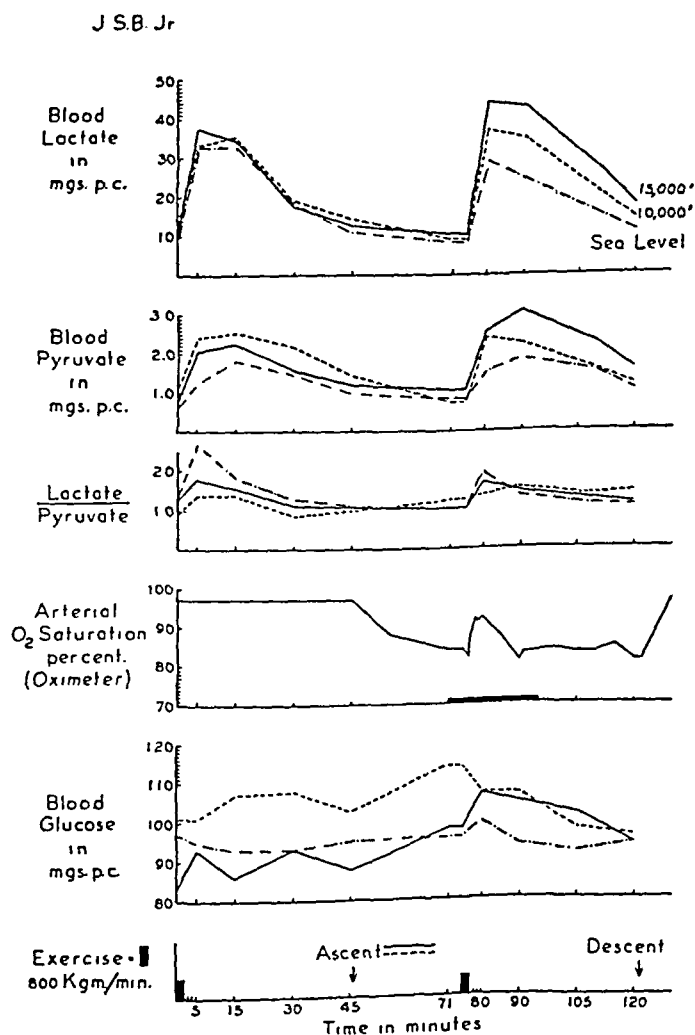


FIG. 3. SUBJECT J. S. B. JR. MALE, AGE 25, HT. 5'10", WT. 138 LBS. WELL-TRAINED CYCLIST. EXERCISE: CYCLE ERGOMETER

Both exercise bouts at sea level, 9/13/43. Second exercise bout at 10,000 feet, 8/16/43. Second exercise bout at 15,000 feet, 8/30/43.

findings in the two untrained subjects, the two trained subjects working at a moderate work load and the two trained subjects working at a heavy work load all showed remarkably close agreement within each pair, the complete experimental record of only one individual in each category will be presented.

Blood lactate. Untrained subject R. G. G. (Figure 2) showed large increases in blood lactate

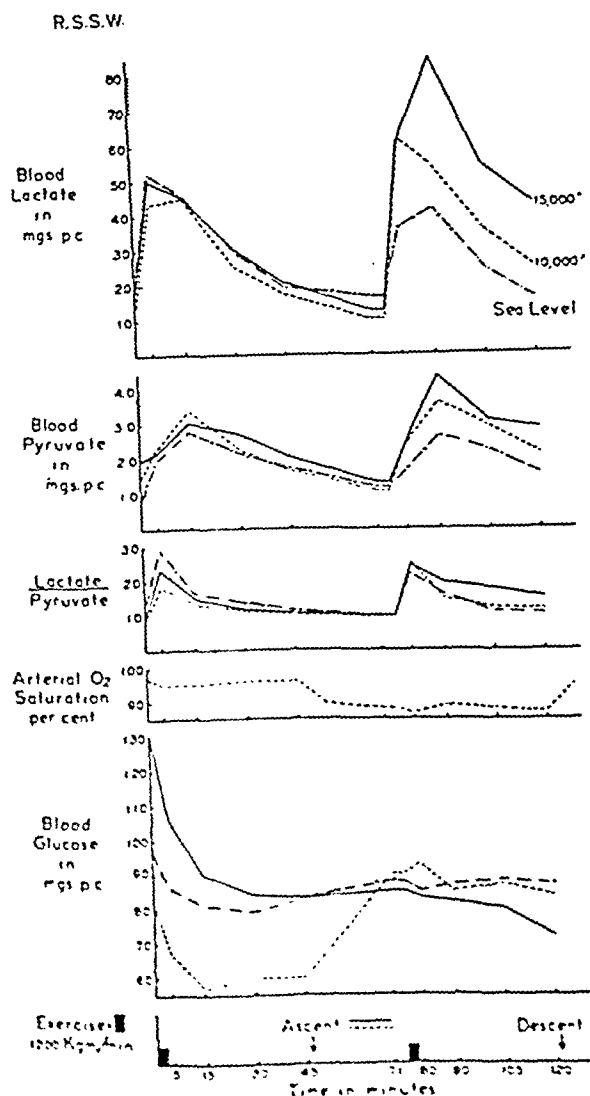


FIG. 4. SUBJECT R. S. S. W. MALE, AGE 23, HT. 5'11", WT. 155 LBS. WELL-TRAINED CYCLIST. EXERCISE: CYCLE ERGOMETER.

Both exercise bouts at sea level, 9/10/43. Second exercise bout at 10,000 feet, 8/27/43. Second exercise bout at 15,000 feet, 9/22/43.

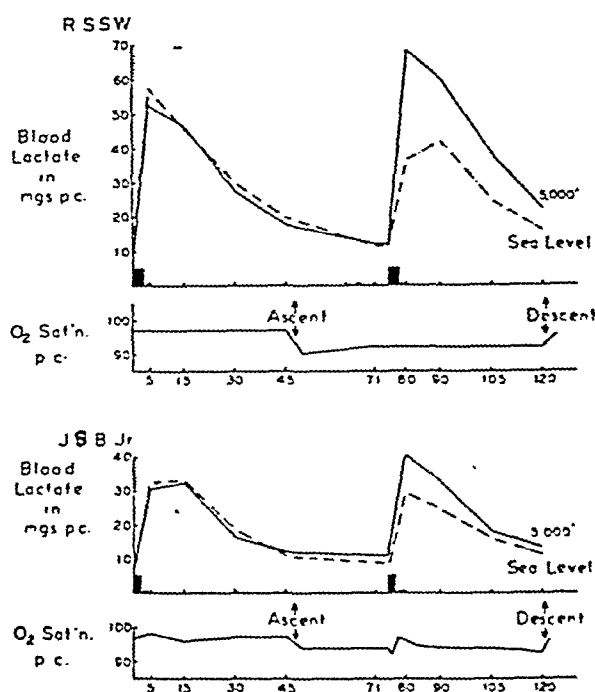


FIG. 5. EFFECT OF EXPOSURE TO 5,000 FEET ON BLOOD LACTATE RESPONSES OF TWO WELL-TRAINED SUBJECTS.

R. S. S. W., 10/13/43. J. S. B. Jr., 10/21/43. For details of previous experiments on these subjects, see Figures 3 and 4, respectively.

after performing a comparatively small amount of work—750 kpm./min. for three minutes. Moreover, no valid comparison may be made between his performance at altitude and at sea level, for an examination of the three curves following his initial exercise bout on each of the test days shows that his record lacks consistency. Whether or not the variability in his response is in part due to training effects cannot be stated with certainty. Although the differences in his blood lactate curves at different altitudes are insignificant, this subject was unable to adapt satisfactorily to a simulated altitude of 15,000 feet, for he was forced by impending syncope to descend to sea level about 18 minutes after his bout of exercise at altitude.

The trained subjects (J. S. B. Jr. and R. S. S. W., Figures 3 and 4, respectively) show a remarkably consistent set of lactate curves following the initial bout of exercise and unequivocal differences in response at different altitudes. A comparison of the record of R. S. S. W. (Figure 4) with that of J. S. B. Jr. (Figure 3) shows that the differences between lactate curves at different altitudes

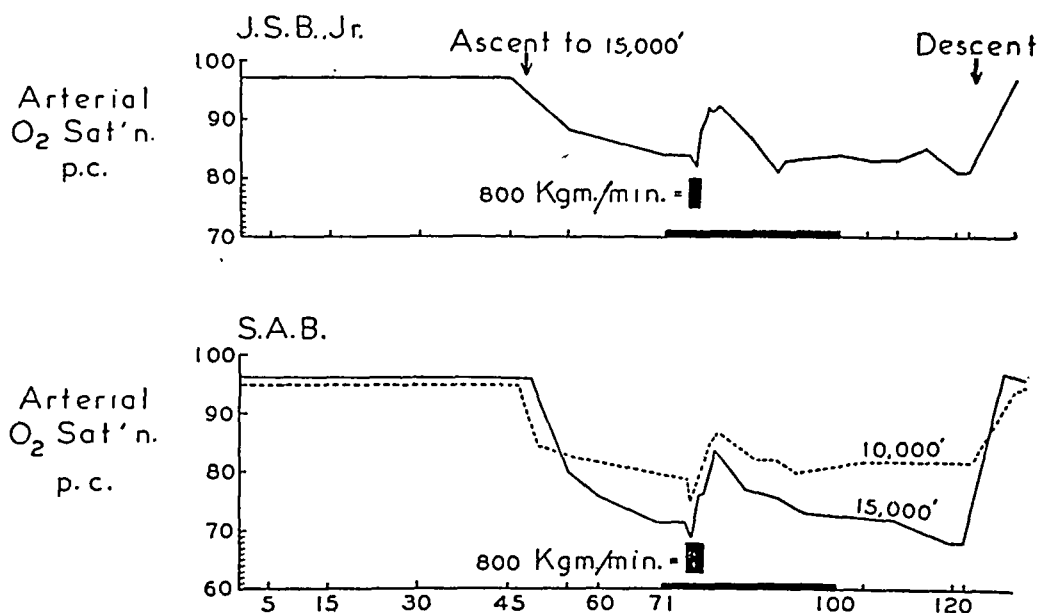


FIG. 6. EFFECT OF EXERCISE UPON THE ARTERIAL OXYGEN SATURATION OF TWO SUBJECTS AT ALTITUDE

Oximeter readings were taken every 10 seconds during time indicated by black bar on time scale.

at sea level are more striking when heavier work loads are done. Presumably this phenomenon has a work-load extinction point since resting blood levels are nearly the same at 15,000 feet as they are at sea level. However, work loads of less than 750 kgm./min. were not used in this series of experiments.

Three well-trained subjects showed unmistakable upward displacement of the blood lactate curves following exercise at 5,000 feet (Figure 5). In all three cases the patterns of response were very similar to those which had previously been observed in the 10,000-foot experiments. Elevated blood lactate curves were accompanied by corresponding changes in the blood pyruvate levels and in the pulse rates following exercise.

Blood pyruvate. In general, the blood pyruvate curves were similar to the homologous blood lactate curves of the individual subjects. Training effects were apparent in the poorly trained subjects and a quite definite stratification was observed at the different altitudes in the four well-trained subjects. However, the pyruvate changes on different occasions at sea level did not show the same degree of consistency found in the corresponding lactate curves, and the pyruvate contrasts with varying degrees of anoxia were not as

sharp as were those of the concurrent blood lactate patterns. It must be emphasized here that the method of Lu was designed to determine alpha keto acids as pyruvate, and that fluctuations in the levels of alpha keto acids other than pyruvate may conceivably occur under the conditions of those experiments. In any case, pyruvate undoubtedly constitutes all but a small portion of the keto acids estimated.

In confirmation of the reports of other investigators, it was found that the peak of the pyruvate curve almost invariably occurred about 15 minutes after the beginning of the exercise period, whereas the truncated configuration of the lactate curves suggests that their peaks occurred most often at about the tenth minute. Since it has been found by Bueding and Goldfarb (10) that the injection of salts of lactic acid results in an appreciable rise in blood pyruvate, the delayed pyruvate peak may be an expression of the re-oxidation of accumulated lactic acid to pyruvic acid.

The *lactate-pyruvate ratio* was suggested as an index of anoxia by Friedemann (11). This ratio has its greatest significance when it is determined at the time of maximal anoxia, and it is of some interest that the peak values for the ratio were almost invariably observed to occur five minutes

after the beginning of exercise. Although the ratio of lactate to pyruvate remained high throughout the experiment in three of the four well-trained subjects at 15,000 feet, curves of the ratio do not show the orderly arrangements characteristic of the lactate curves, nor do they correlate well with the state of athletic training of the individual subjects.

Arterial oxygen saturation. Arterial oxygen saturation was estimated by means of the Millikan oximeter. At saturations between 70 per cent and 85 per cent it was found that the galvanometer frequently oscillated slowly over a range of as much as 10 per cent. In such cases the mean deflection was used as the basis of the graphic records in Figures 3 to 5.

There was a noteworthy variation in the degree of arterial unsaturation produced in different subjects by the same degree of reduction in atmospheric pressure. For example, the estimated arterial saturation of J. S. B. Jr. at 15,000 feet was about the same as that of S. A. B. at 10,000 feet.

On five separate occasions definite upward displacement of the blood lactate curve was observed in individuals whose arterial saturation (as estimated by the oximeter) was 90 per cent or above. During two of these experiments readings were rarely below 94 per cent. This is arresting in view of the frequently made statement that symptoms of anoxia are not detectable in human subjects whose arterial oxygen saturation remains above 90 per cent.

The oximeter records of J. S. B. Jr. and S. A. B. afford an opportunity to study the arterial oxygen saturation during exercise. In these experiments oximeter readings were taken every ten seconds during and after exercise. All of the records show a small initial fall in saturation, followed by a rise over the starting level, the peak occurring about five minutes after the beginning of exercise. Subsequently, there is a return to the resting level (See Figure 6).

Blood glucose. Blood glucose concentrations following exercise at sea level and under varying degrees of anoxia were extremely variable. Often exercise was accompanied by a moderate degree of hypoglycemia; but almost as often, the bout of exercise was followed by a small rise in blood sugar. The bizarre patterns described by the variations in blood glucose concentration were in

TABLE I

Effect of hypoxia on pulse rise following measured exercise
Chemical data for these subjects are given in Figures 2 to 4.

Subject	Altitude	Before exercise	Pulse 4 min. after exercise	45 min. after exercise
R. G. G.	Sea level	92	100	90
	10,000'	88	120	88
	15,000'	90	150	90
J. S. B. Jr.	Sea level	74	122	74
	10,000'	96	130	94
	15,000'	86	140	94
R. S. S. W.	Sea level	72	128	68
	10,000'	66	136	70
	15,000'	74	158	88

marked contrast to the orderly profiles of the blood lactate curves.

Amino acid nitrogen and hematocrit. Amino acid nitrogen analyses were performed on the acid tungstate filtrates that were used for glucose analysis. It was found that variations in the blood amino acid nitrogen were very small, and that no increase in the concentration of this blood constituent occurred at 15,000 feet.

The very small apparent rises in amino acid nitrogen frequently seen after exercise may be related to the hemoconcentration which is almost invariably seen following a burst of muscular work, for the ratio of amino acid nitrogen in red cells and plasma has been found in this laboratory to be about 3.2 to 1. The hemoconcentration of exercise has been studied by Keys and Taylor (12), who regard it as true hemoconcentration, and not the result of an increased number of circulating red blood cells. It is of some interest that hemoconcentration at altitude did not appear to be greater, or of longer duration, than it was at sea level.

Pulse. The pulse rate immediately after exercise at altitude was higher than it was at sea level. In the four trained subjects the maximal pulse rates varied directly with the maximal lactate concentrations in 11 out of 12 experiments (see Table I).

II. STUDIES RELATING TO THE MECHANISM OF THE BLOOD LACTATE RESPONSE TO EXERCISE AT LOW BAROMETRIC PRESSURES

Bang (2) concluded that the predominant factor in the accumulation of lactate in the blood was

overproduction of lactic acid by comparatively anaerobic working muscle during the first few minutes of exercise. It is postulated here that the differences in lactate accumulation after measured exercise at sea level and at 15,000 feet may also be related to events that occur during the transition from rest to work, rather than to any serious impairment of the oxidative removal of lactate from the blood. This section is devoted to a description of the results of three experiments which tend to support this hypothesis.

A. A comparison of the disappearance rate of lactate at sea level and at 15,000 feet

A comparison of the disappearance rates of lactate after exercise at sea level and at 15,000 feet was not considered feasible because the peak concentration of lactate at 15,000 feet was frequently twice that at sea level. In the case of subjects who did amounts of work comparable to those done by P. T. and DeW. B. in the present study, the difference in lactate concentration at sea level and at 15,000 feet at the summits of the respective curves was 30 to 50 mgs. per cent. It is well known that the rate at which many substances disappear from the blood is in part dependent upon their initial concentration. It was therefore necessary to design an experiment to compare lactate disappearance at sea level and at 15,000 feet; that is to say, an experiment in which the starting lactate levels would be as nearly alike as possible.

At first, it was thought that this could be accomplished by injecting the same amount of a lactic acid salt on two occasions, once at sea level and once at 15,000 feet, and determining the disappearance rates under the two conditions. Accordingly, subject J. L. G. was given 16.4 gms. of racemic Sodium Lactate (Lilly) in 960 cc. of water over a period of 21 minutes. As the subject received the last of the infusion the chamber was decompressed to a simulated altitude of 15,000 feet at the rate of 5,000 feet per minute. After four minutes at 15,000 feet the subject suddenly became quite pale, said he "felt funny," and promptly lost consciousness. After a period of apnea lasting about 10 seconds, 10 per cent carbon dioxide in 90 per cent oxygen was administered by oxygen mask and respirations were resumed almost immediately. When he regained consciousness after

about 45 seconds, the subject was very pale and his pulse was rapid and thready. The experiment was therefore terminated. It is suggested that syncope in this case was due to the additive effects of hyperventilation alkalosis superimposed on a pre-existing, and probably, progressively developing, sodium lactate alkalosis. In view of the evident risk involved, this experimental approach was discarded.

Another technique of securing identical starting levels of blood lactate was adopted. In addition to dispensing with an intravenous infusion, this method had the advantage of introducing the natural isomer of lactic acid into the blood, rather than a racemic mixture. The subject exercised for three minutes on the cycle ergometer at sea level and the blood lactate curve at sea level was followed over a period of 60 minutes. On another day, under as nearly the same conditions as possible, he performed the same amount of exercise at sea level, remaining at sea level for 9 minutes. Between 9 and 12 minutes after the beginning of the exercise bout the chamber was decompressed to a simulated altitude of 15,000 feet, and the recovery curve was determined at that altitude during the subsequent 48 minutes, the 10-minute sample having been obtained during decompression. Two such experiments were completed; the results are presented graphically in Figure 7. Subject P. T., who was a highly trained athlete whose day to day lactate response curves to the same work load were practically identical, showed almost no difference in lactate or pyruvate disappearance rates at the two altitudes. Poorly-trained subject DeW. B., however, showed a slightly slower rate of disappearance of both lactate and pyruvate at 15,000 feet than at sea level. Other sea level control curves on the latter subject, however, deviated from each other by 2 to 5 mgs. per cent, which tends to detract from the significance of the difference found here.

B. Effect of breathing 100 per cent oxygen on blood lactate and pyruvate responses to exercise at sea level

A comparison was made of the contour of the blood lactate and pyruvate responses to measured exercise in a well-trained subject, P. T., who breathed 100 per cent oxygen during exercise on

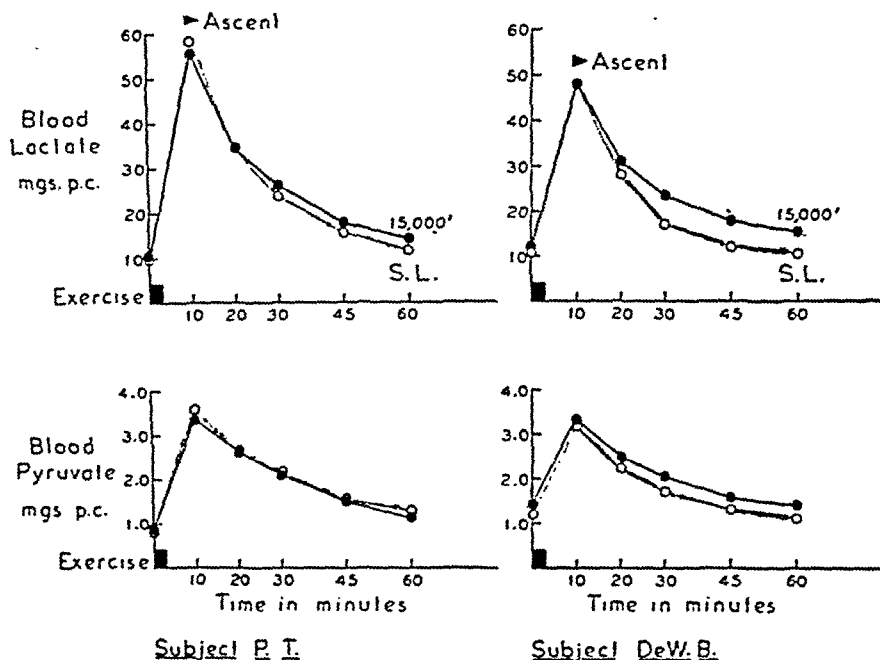


FIG. 7. LACTATE DISAPPEARANCE AT 15,000 FEET AND AT SEA LEVEL

Subject P. T., well-trained. Subject DeW. B., poorly trained. On the day marked 15,000 feet the chamber was decompressed to that altitude between the 9th and 12th minutes of elapsed time.

one occasion. This subject performed so consistently that blood lactate curves obtained on five different occasions at the same work load were almost precisely superimposable. Figure 8 shows the effect of breathing 100 per cent oxygen during exercise, and room air during recovery. The maximum deviation from the control curve is about 8 mgs. per cent, which, in the case of this subject, is regarded as highly significant.

Similar experiments were done on two other subjects, DeW. B. and J. T., but the results were inconclusive. These subjects were in poor athletic training, and it was found that the blood lactate and pyruvate responses to standard exercise during oxygen breathing were not significantly different from the rather variable responses of these subjects obtained during air breathing.

C. Effect of intravenous infusion of glucose on blood lactate and pyruvate at sea level and at 15,000 feet

Another type of experiment was designed to evaluate the importance of the exercise bout as compared with the recovery period in the lactate

response to exercise at altitude. It is well known that ingestion or injection of glucose is followed by transitory elevation of the lactic and pyruvic acid concentrations of the blood of many species (for example, see Bueding and Goldfarb, [10]). If the rate of removal of lactic acid from the blood is an important determinant of the contour of the lactate curve at altitude, it was suggested that glucose infusion at altitude might result in a higher and longer sustained lactacidemia than at sea level. That this is not the case is shown by the following experiments.

Subjects J. L. G. and E. P. were studied as follows: 100 gms. of glucose in 700 cc. of water were given intravenously over a period of about 20 minutes. On one day, a sea level control experiment was performed; on another day, the chamber was decompressed to a simulated altitude of 15,000 feet at the end of the infusion. Periodic blood samples were taken during the two hours following the termination of the infusion and the subject was observed. Blood glucose, lactate, and pyruvate were estimated. Experiments on the same subject were separated by three days, and, in

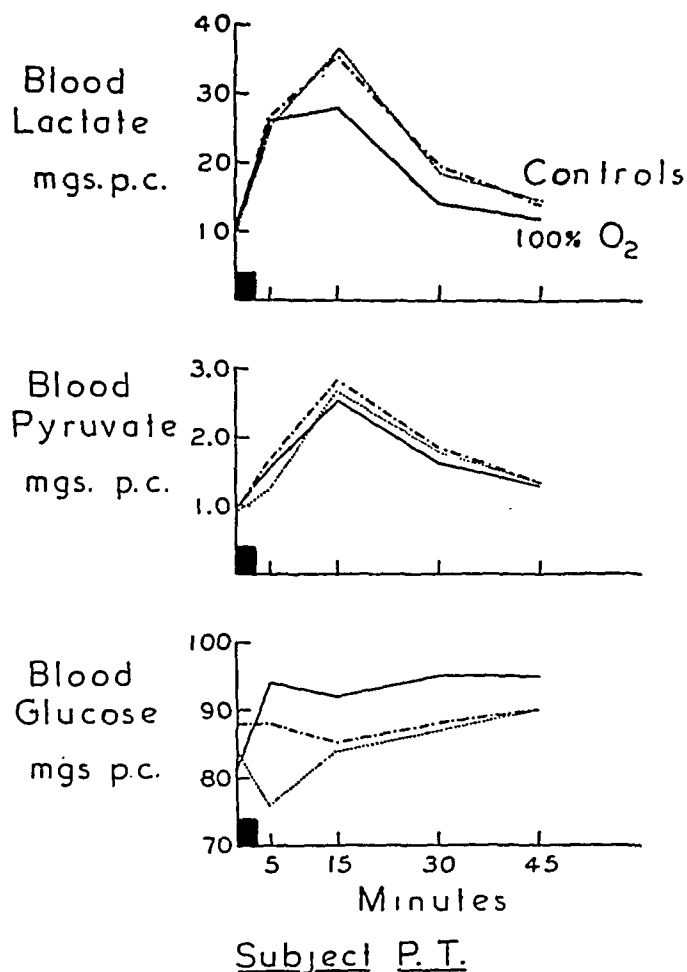


FIG. 8. EFFECT OF BREATHING 100 PER CENT OXYGEN ON BLOOD LACTATE RESPONSE TO MEASURED EXERCISE IN A WELL-TRAINED SUBJECT

Oxygen was administered only during the three-minute exercise bout.

the case of J. L. G., the experiment at altitude was carried out before the sea level control study, whereas this order was reversed in the experiments on E. P.

The results are given graphically in Figure 9. It is immediately apparent that the order of magnitude of the rise in lactate and pyruvate provoked by the glucose infusion is the same at 15,000 feet as at sea level; in both instances the change in lactate is of about the same extent as that found by Bueding and Goldfarb (10) in their sea level experiments; namely, about 10 mgs. per cent.

The blood glucose in both subjects achieved levels of 550 mgs. per cent or more when the estimation was made on samples taken ten minutes after the end of the infusion. In each subject, a definite hypoglycemic reaction (paresthesias,

sweating, palpitations, faintness and hunger) occurred at about 90 minutes in the 15,000-feet experiment. E. P.'s blood glucose shortly after the onset of his hypoglycemic reaction was about 65 mgs. per cent; in J. L. G. the corresponding value was about 60 mgs. per cent. The former subject experienced a similar reaction during the sea level experiment, but it occurred about 30 minutes later than it did at altitude. J. L. G. had no hypoglycemic episode during the sea level experiment.

DISCUSSION

The results of the experiments on subjects in a poor state of athletic training suggest that, as Robinson and Harmon (13) have indicated, each individual has a certain maximum capacity for accumulating lactic acid in the blood. The muscles of the untrained subjects can be regarded as having been so hypoxic at sea level that lowering the arterial oxygen saturation produced no evident additional embarrassment. In the case of the two untrained individuals who did deep knee bends (Figure 1), approximately the same amount of lactic acid was accumulated at 15,000 feet as at sea level, although one-third less work was done in the former experiment.

The well-trained individuals worked well within their respective capacities. The complex series of responses that occur when such an individual begins to exercise were operating at such a high level of efficiency that arterial oxygen saturation was revealed as a limiting factor in the transition from work to rest. Thus, comparatively small changes in that parameter were reflected in easily measurable differences in lactate accumulation.

The fact that a second bout of exercise at sea level regularly results in a smaller lactate rise than the first suggests the occurrence of a short-term adaptation reminiscent of the Staub-Traugott effect seen following the administration of repeated doses of glucose. The lactate response may be regarded as an expression of the total response of the organism to the change from the resting to the working state. This response includes intracellular components which are related to the actual conversion of chemical into contractile energy, and a complex of reactions that occur outside of the muscle cells, including the respiratory

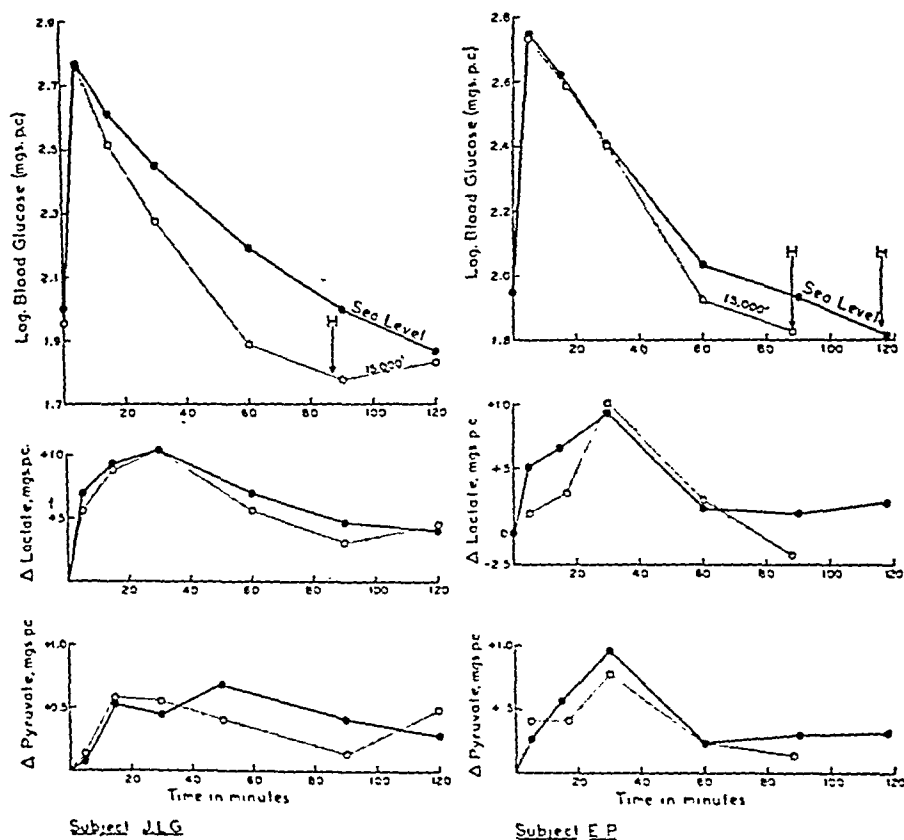


FIG. 9. EFFECT OF GLUCOSE INFUSION ON BLOOD LACTATE AND PYRUVATE AT 15,000 FEET AND AT SEA LEVEL

"H" signifies hypoglycemic episodes.

and cardiovascular reflexes that are initiated by the beginning of exercise. Both intramuscular and extramuscular events may be involved in the fact that the muscle, having recently performed a bout of exercise, begins its second bout with an apparent advantage.

Failure of lactate/pyruvate ratios to correlate with the degree of anoxia in working subjects is in agreement with the findings of Bay and his colleagues (14). These workers state that decrease of blood pyruvate does not truly picture return to aerobic conditions since the disappearance of pyruvate may be due to a variety of reactions, including condensation. From the data presented here it can only be inferred that increases in pyruvate concentrations, within the limits of the greater error involved in measuring smaller amounts of material, are approximately proportional to corresponding increases in lactate concentration.

That impairment of lactate removal is not a significant factor in the upward displacement of the lactate exercise response curve is suggested by the three types of experiment designed to analyze this point. On the basis of the experiments reported here it would have been impossible to demonstrate such an impairment at 5,000 or 10,000 feet. Yet, at those altitudes upward displacement of the blood lactate curve after measured exercise occurred in three individuals. That the exercise period is critical in this displacement is indicated by the fact that there is no significant elevation of the lactate curve when it is produced by a technique not involving exercise; i.e., by glucose infusion. Added to these arguments is the fact that breathing 100 per cent oxygen during the three-minute exercise period produced a clear-cut expression of the lactate response curve in one highly trained athlete.

After these experiments were completed an ex-

portunity arose for demonstrating a practical application of the lactate response test in another circumstance, anemic anoxia. When the feasibility was considered by the Chemical Warfare Service of protecting men prophylactically against cyanide poisoning by inducing methemoglobinemia, it was necessary to study the effect of methemoglobinemia upon human performance. It was shown by means of the exercise-lactate test (15) that sufficient methemoglobinemia to provide moderate protection against cyanide intoxication produced a definite impairment of oxygenation of human muscle working at moderately heavy work loads.

SUMMARY

The lactic acid concentration of the blood after measured exercise was higher at low barometric pressure than at sea level pressure. Upward displacement of the lactate curve was found at a simulated altitude as low as 5,000 feet in three well-trained subjects.

Two subjects in a poor state of athletic training did not show the same orderly, progressive elevation of lactate response curves with increasing hypoxia demonstrated in four well-trained subjects.

In addition to changes in lactate concentration, fluctuations in pyruvate, glucose and amino acid nitrogen concentrations, and in hematocrit, arterial oxygen saturation and pulse rate are described.

From studies on the rate of disappearance of lactate from the blood stream at low barometric pressure and at sea level; on breathing 100 per cent oxygen during exercise; and on the blood lactate rise at altitude following glucose infusion, it is concluded that overproduction of lactate during exercise is the critical determinant of the response described above. Interference with the mechanisms involved in the removal of lactate from the bloodstream does not contribute significantly to the lactate response at the simulated altitudes studied.

ACKNOWLEDGMENT

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FACTORS AFFECTING THE APPEARANCE AND PERSISTENCE OF VISIBLE CUTANEOUS REACTIVE HYPEREMIA IN MAN¹

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Changes in cutaneous reactive hyperemia have been used successfully to identify certain local (1, 2) and general (3 to 5) abnormalities of the circulation. More recently it has been observed that cutaneous reactive hyperemia is altered conspicuously in normal subjects by vigorous exercise, particularly as the circulatory stress of exhaustion appears (6). Under these conditions venous pressure, cutaneous blood flow, and skin temperature were changing simultaneously and rapidly. Hence it was difficult to understand the mechanism by which cutaneous reactive hyperemia may be altered without more information on the factors which modify it in the normal subject under experimental conditions.

This account describes the effects of environmental temperature, skin temperature, elevation of the arm, venous pressure, external pressure and epinephrine iontophoresis, on the appearance and persistence of visible cutaneous reactive hyperemia. It indicates certain factors which should be kept constant when reactive hyperemia is used as a test to identify changes in cutaneous circulation.

METHODS

Cutaneous reactive hyperemia was produced locally on the horizontal volar surface of the forearm by placing a weighted plastic ring on the skin for sufficient time to produce, after removal of the ring, a distinct annular redness of the skin as described by DiPalma *et al.* (7). The plastic ring itself had an outer diameter of 3.5 cm., an inner diameter of 2.5 cm., and a total surface area of 5 sq. cm. It was attached to the under surface of a 500 gm. weight. When resting freely on the skin the weight applied to the skin immediately beneath the ring a pressure equivalent to 73 mm. Hg, i.e., sufficient to collapse

the minute vessels and to arrest blood flow (8). Direct observations through the transparent ring showed that the skin was completely blanched by this pressure. In this account the definitions and criteria described by DiPalma *et al.* (7) will be followed.

"Threshold time" or "threshold" is the least duration of application in seconds required to produce a complete hyperemic ring having a width equal to the surface of the plastic ring. Applications lasting less than "threshold time" produced no visible hyperemia or a patchy incomplete area of hyperemia which was either narrower or broader than the ring itself. The end point is undeniably subjective and its estimation requires certain practice, but with experience, and under constant conditions, readings can be duplicated within plus or minus 20 per cent over periods of several hours. This subjective element was finally reduced to a minimum by having one observer make readings without knowing the exact duration of the preceding occlusion, the latter being recorded by a second observer. The juxtaposition of normal skin just inside and outside the ring of hyperemia made comparisons simpler, more quantitative and more rapid than was possible with the earlier method of Lewis and Grant (1, 9), in which the color of one forearm had to be compared with that of the opposite forearm.

"Clearing time" is defined (7) as the time, in seconds, for complete disappearance of that reactive hyperemia which has been produced by an occlusion of threshold duration. Repeated readings of clearing time over short periods usually agreed within plus or minus 25 per cent, but occasionally varied more widely. For this reason it was found impossible to attribute as much significance to single estimations of clearing time as to single determinations of threshold.

Uniform lighting, with minimum heating, was obtained by using either (a) large 40-watt "Daylight" fluorescent bulbs at a distance to illuminate the whole forearm or (b) two small 4-watt bulbs, 5 inches long, one on each side of the forearm under reflectors.

Environmental temperature was kept constant in most observations to within $\pm 0.5^\circ$ C. by using a constant temperature room. Skin temperatures were measured by means of iron-constantan silver-soldered thermal junctions. The thermal junctions were at first applied under adhesive tape, which covered the junction, but later a springy celluloid mounting was found more satisfactory because the adhesive tape often covered only the wires leading to the junctions while the junctions themselves rested against the skin without any pressure.

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² Alexander McPhedran Fellow from the Department of Medicine, University of Toronto.

³ Student Research Fellow, Life Insurance Medical Research Fund.

Venous pressure was elevated in the forearm by means of a wide (15 cm.) pneumatic cuff, containing a rubber bag long enough to encircle the upper arm completely. Instantaneous inflation was assured by connecting the pneumatic cuff to a large reservoir of air, previously brought to the desired pressure. Direct readings of venous pressure in several instances verified the equality of venous pressure and pressure in the pneumatic cuff.

OBSERVATIONS

I. Comparison of gross reactive hyperemia (Lewis and Grant, [9]), with localized reactive hyperemia (DiPalma et al., [2])

Lewis and Grant (9) observed that occluding the circulation to the entire forearm for one or more minutes produced a reactive hyperemia which persisted, after release of the circulation, for a period of one-half to two-thirds the duration of the occlusion. Thus occlusion for one minute produced a reactive hyperemia which persisted visually for 40 to 65 seconds, and plethysmographically for about 25 seconds. It is interesting that for both brief and prolonged occlusions, the visible redness of the skin appeared to be a more sensitive indicator of persisting mild reactive hyperemia than the plethysmographic measurement of total forearm volume, which included the vessels of both muscle and skin.

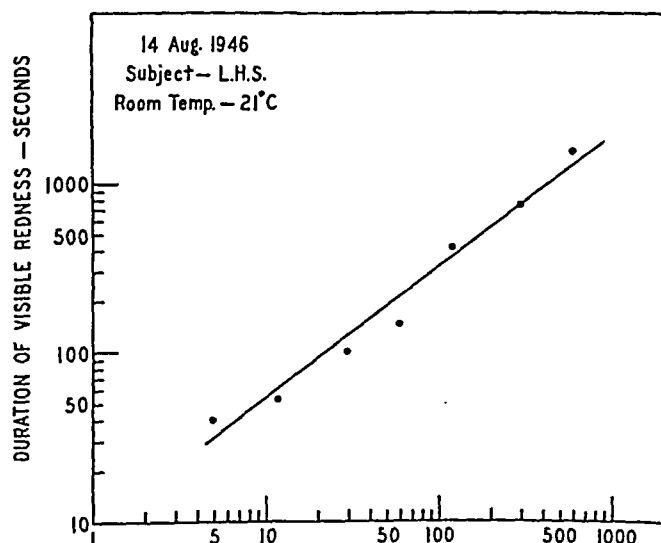


FIG. 1. LOGARITHMIC PLOT SHOWING RELATION BETWEEN DURATION OF OCCLUSION AND DURATION OF REACTIVE HYPEREMIA, IN ONE SUBJECT

Similar relation was observed in 48 experiments on three subjects at room temperatures from 17° to 37° C. and with congesting pressures from 0 to 60 cm. water.

Figure 1 shows that, in localized reactive hyperemia also, increasing the time of occlusion increased the duration of the local hyperemic response. With the arm at or below heart level reactive hyperemia was absent until the time of occlusion was increased to 5 seconds, which was therefore the "threshold" in this instance. Above this threshold visible reddening of the skin lasted from two to eight times as long as the preceding occlusion. This prolonged effect of local occlusion by pressure of the ring can be ascribed to several factors. (1) Because normal and hyperemic skin are adjacent to each other the contrast afforded by the ring of hyperemia is more accurate than comparison of one forearm with the other. (2) The ring not only arrests blood flow but, through external pressure, also evacuates blood from the minute vessel whereas the inflation of a cuff on the upper arm, as done by Lewis and Grant (9), leaves within all the minute vessels a certain amount of oxygenated blood and hence, for a given time of occlusion, produces less hypoxia than does the weighted ring. Of far less importance, but still conceivably contributory, may be (3) arrest of diffusion of atmospheric oxygen through the skin (Goldschmidt and McGlone, [10]) while compressed by the ring, and (4) transitory increase of vasoconstrictor tone due to pain produced when an occluding cuff is used (Abramson et al., [11]).

The relative accuracy of long and short applications of the ring under several experimental conditions showed that the briefer and repeated determinations of "threshold time" were more accurate and sensitive than the longer applications, e.g., 5 or 10 minutes. The latter, in fact, produced depressions in the skin which made accurate readings more difficult. Hence for the remaining studies only threshold values were determined. The close relation between duration of occlusion and duration of reactive hyperemia showed, however, that this local response is essentially similar to that described by Lewis and Grant (9) following the release of an occluding cuff on the upper arm.

II. Effect of environmental and cutaneous temperatures on reactive hyperemia

Intensification of reactive hyperemia by warming the skin in a water bath was described by

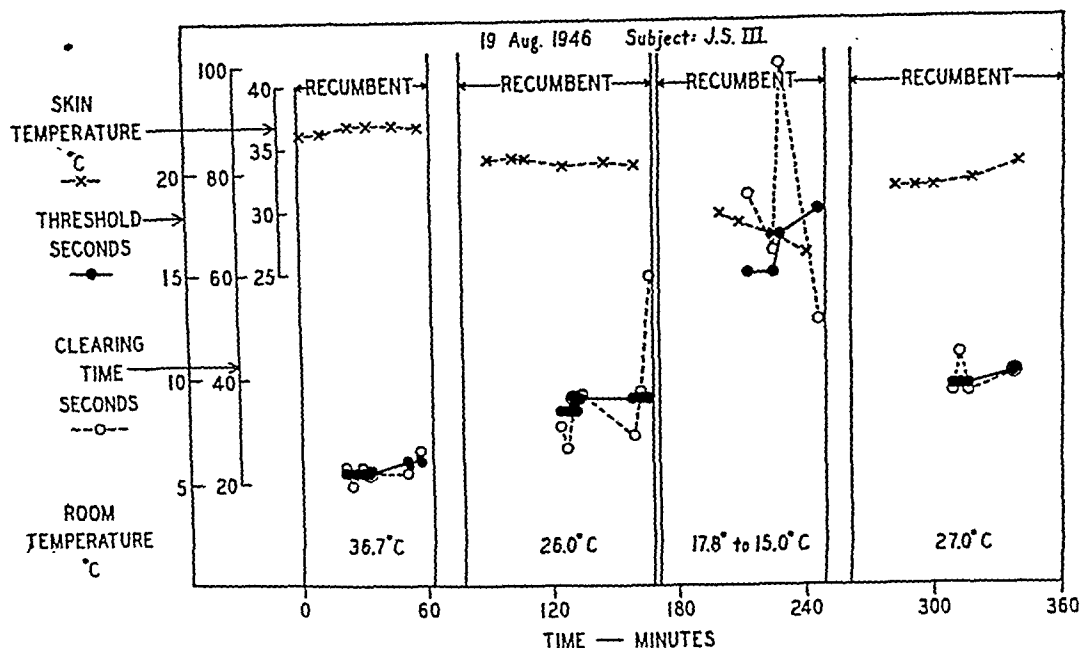


FIG. 2. CHART SHOWING EFFECTS OF ENVIRONMENTAL TEMPERATURE ON THRESHOLD (SOLID DOTS AND LINES) AND ON CLEARING TIME (CIRCLES AND DOTTED LINES) OF REACTIVE HYPEREMIA

Skin temperature is shown by crosses. Subject recumbent with forearm at heart level during observations but moving about between observations. Thermal junctions in this experiment covered with one layer of adhesive tape.

Lewis and Grant (9). This relation was defined more quantitatively by determining thresholds and clearing times at environmental temperatures ranging from 17° to 37° C. and skin temperatures ranging from 26° to 37° C. The results of a typical experiment are shown in Figure 2.

At zero time the subject, dressed only in shorts, had been recumbent for 30 minutes in the controlled temperature room at 36.7° C. Thresholds and clearing times, skin temperatures and room temperatures were recorded for 30 or more minutes. The subject was then allowed to move about for about 10 minutes. The room temperature was lowered to 26.0° C., the subject again lay down for 30 minutes and observations were made at this new temperature. In each experiment room temperatures of 37°, 27°, and 17° C. were used in ascending or descending order with a final observation at 27° C. As indicated in Figure 2 threshold and clearing times became progressively longer as environmental temperature was reduced. Although observations on three male subjects, aged 22 to 33 years, yielded results entirely

similar to those in Figure 2, charting threshold and clearing times against environmental temperature showed wide variations.

However, when threshold and clearing times were charted against skin temperature, the effect of tissue temperature on reactive hyperemia became much clearer as indicated in Figure 3. Though not always evident in a single subject, the average curves indicate clearly that reducing skin temperature by 5° C. from 37° to 32° C., increased average threshold and clearing times by 50 per cent or less, whereas a like reduction of 5° C., but from 32° to 27° C., increased both threshold and clearing times three- or four-fold. Under "Discussion" reasons will be given for believing that at skin temperatures above 32° C. reactive hyperemia is determined almost solely by accumulated metabolites, whereas at skin temperatures below 32° C. the behavior of the cutaneous vessels in reactive hyperemia depends upon the balance between centrally induced vasoconstrictor tone and the local and opposing vasodilator effect of metabolites.

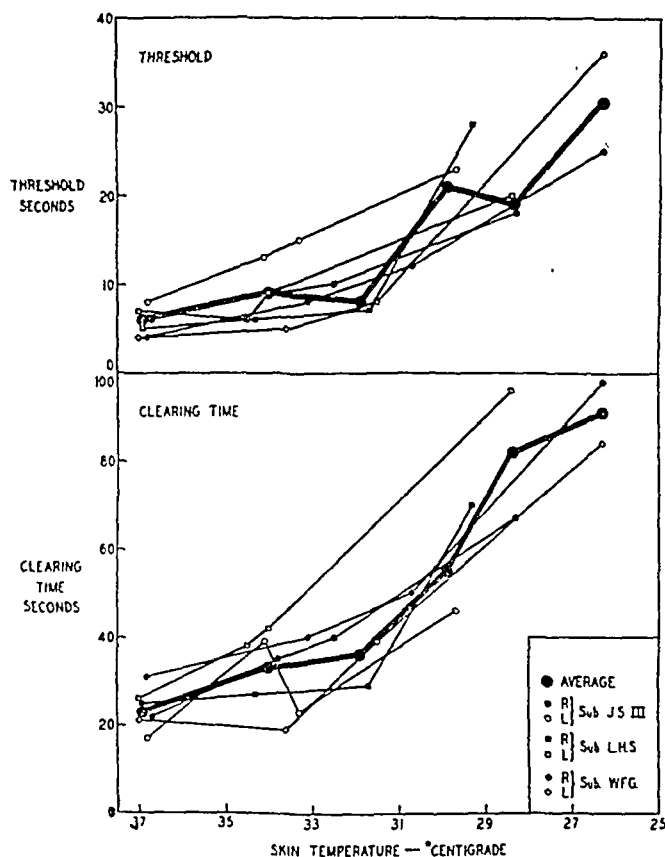


FIG. 3. CHART SHOWING THE RELATION BETWEEN SKIN TEMPERATURE AND THRESHOLD (ABOVE) AND CLEARING TIME (BELOW)

Includes observations on three subjects made on right and left forearms independently by two observers. The five lines represent individual observations. The large dots and heavy lines indicate averages along both coordinates of all observations falling within each interval of 2° C. from 37° to 26° C.

III. Effect of increased venous pressure on reactive hyperemia

It was also found that venous pressure had marked effects on both threshold and clearing time, *i.e.*, on the ease with which visible and persistent reactive hyperemia could be produced. The results of a typical experiment are shown in Figure 4. At zero time the subject had been recumbent for 30 minutes, with the forearm at heart level. Threshold and clearing times having been relatively constant for 30 minutes, venous pressure was elevated suddenly to 40 cm. H₂O. Threshold fell gradually over 5 to 20 minutes to one-fourth the control value and clearing time became, for a few readings, three times greater, finally stabilizing at about twice the control value.

While clearing time occasionally rose briefly to a peak as shown to the left in Figure 4, it was more usually elevated merely to a plateau, as shown in the middle and to the right in Figure 4. Results observed in seven subjects are summarized in Table I. Elevating venous pressure to 20 cm. H₂O lowered threshold in five of seven subjects and increased clearing time in all. Venous pressures of 30 or more cm. H₂O reduced threshold and raised clearing time in all subjects. On the average these effects became more conspicuous as venous pressure increased but in individual subjects this was not always the case (Table I).

Measurements of skin temperature demonstrated the slight cooling of the skin ordinarily found during venous congestion but these changes were far too small to explain the marked changes in reactive hyperemia. A more delicate differential thermopile was also used to detect changes in cutaneous blood flow. Congesting pressures of 20 and 30 cm. H₂O reduced blood flow by a barely measurable amount. This reduction seemed far too small to explain the markedly reduced threshold and increased clearing time, though the action of gradually accumulating metabolites cannot be excluded entirely.

Subsequent observations on the effects of position and of external pressure led to the conclusion that the appearance and duration of visible reactive hyperemia are greatly modified by the degree

TABLE I

Effect of venous pressure on visible reactive hyperemia

Venous pressure	Change of threshold per cent of control			Change of clearing time per cent of control		
	20 cm. H ₂ O	30 cm. H ₂ O	40 cm. H ₂ O	20 cm. H ₂ O	30 cm. H ₂ O	40 cm. H ₂ O
Subject	per cent	per cent	per cent	per cent	per cent	per cent
DiP.	0	-38	-80	+33	+50	+16
A. C. B.	-44	-61	-74	+55	+110	+140
A. C. B.	0	-20	-20	+50	+66	+78
J. S., III	-42	-39	-44	+78	+68	+43
J. S., III	-40	-40	-40	+67	+46	+69
W. F. G.	-25	-30	-40	+22	+41	+67
L. H. S.	-58	-43	-72	+15	+87	+107
Average change, per cent of control	-30	-39	-53	+46	+67	+74

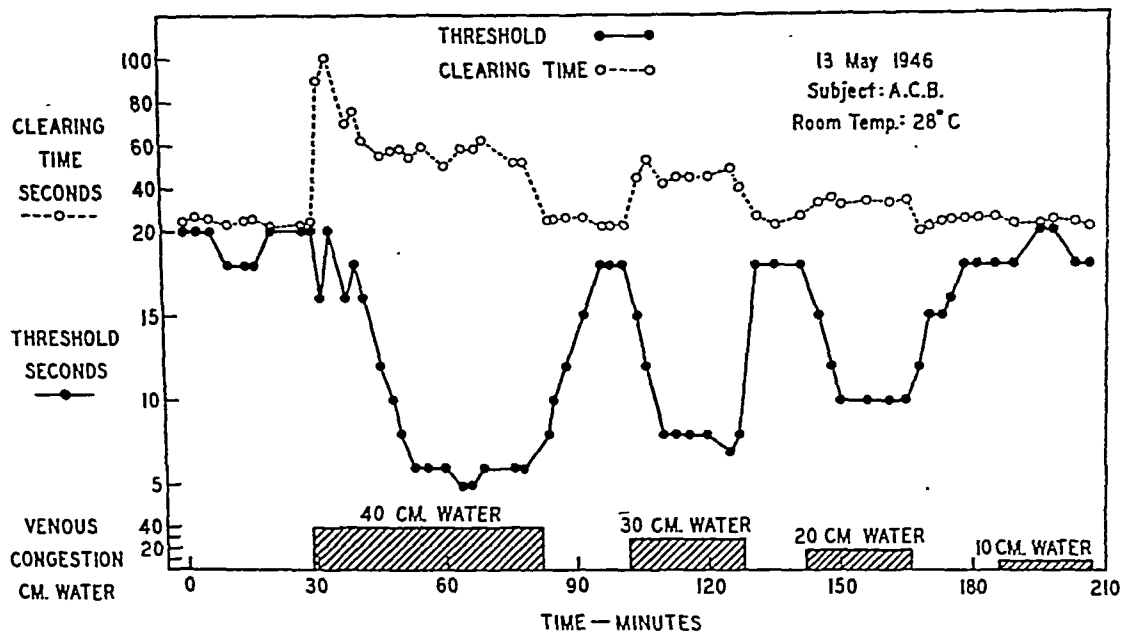


FIG. 4. CHART SHOWING EFFECT OF VENOUS PRESSURE ON THE THRESHOLD AND CLEARING TIME OF REACTIVE HYPEREMIA

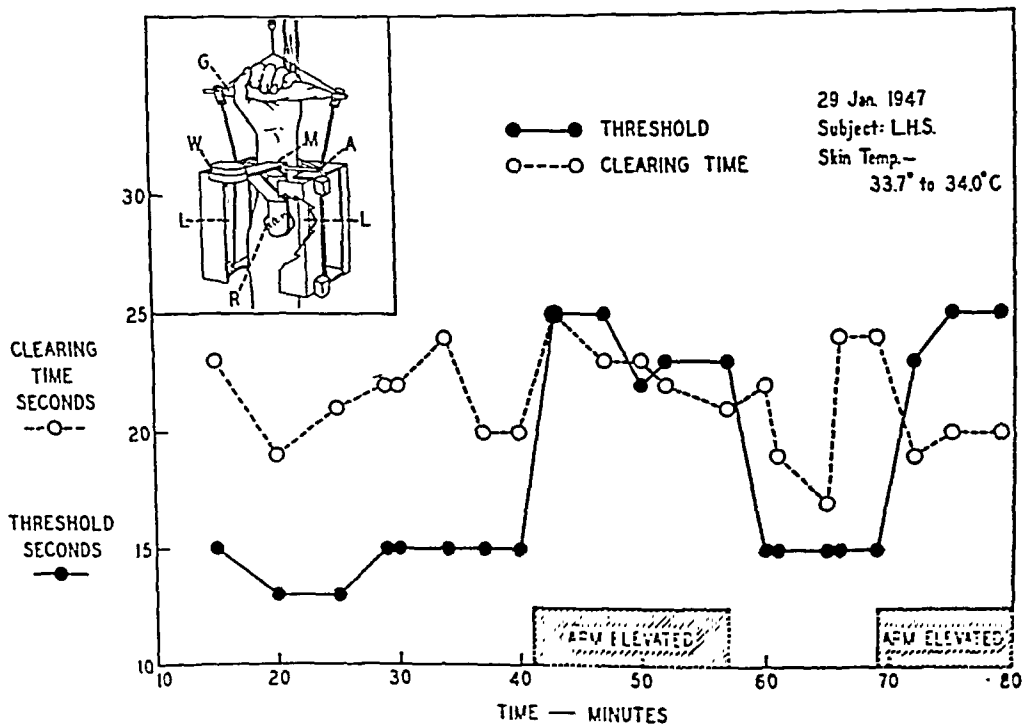


FIG. 5. CHART SHOWING EFFECT OF ELEVATING THE FOREARM ABOVE HEART LEVEL

At zero time subject was recumbent with forearm at side. Forearm elevated as indicated on abscissa, placing test area 30 to 40 cm. above the angle of Louis. In elevated position thresholds and clearing times were determined by means of apparatus shown by inset.

to which the subpapillary venous plexus is distended prior to, and during, reactive hyperemia.

IV. *Effect of elevating the forearm (and reducing venous pressure) on visible reactive hyperemia*

When the volar surface of the forearm was horizontal at heart level or below, the occluding ring could simply be laid on the skin. To test the effect of lowering venous pressure by raising the forearm well above heart level, it was necessary to mount the plastic ring on the right-angled holder shown by the inset of Figure 5.

With the forearm suspended vertically from the handgrip "G," the right-angled mounting "M" moved freely on the axle "A." When this mounting rested firmly against the skin, the 500-gm. weight "W" pressed the ring "R" against the skin of the vertically placed forearm with a pressure of about 73 mm. Hg just as the simple weight and ring did in the horizontal forearm. Two small fluorescent lamps "L" provided uniform lighting in whatever position the forearm might be.

A typical experiment is shown in Figure 5. At heart level, threshold was 13 to 15 seconds and clearing time averaged 22 seconds. Elevating the forearm as far as possible above heart level raised threshold promptly and conspicuously but did not change clearing time significantly. Conversely, lowering the forearm to heart level returned threshold again to the control level. Table II summarizes the results of similar experiments on three subjects. To produce visible hyperemia in the elevated forearm required an occlusion last-

ing 66 per cent longer than in the horizontal forearm. Despite this longer occlusion, however, the clearing time was not significantly changed; *i.e.*, the hyperemia remained visible for the usual period of 20 to 40 seconds in both positions. Changes in skin temperature were again too slight to explain the observed changes in threshold.

V. *Effect of external pressure on reactive hyperemia*

The changes in visible reactive hyperemia produced by venous congestion and change of position suggested that the state of the subpapillary venous plexus, *i.e.*, whether distended or collapsed, influences the intensity of skin color in reactive hyperemia as it does normal skin color (Lewis, [12]). Hence external pressure was used to collapse the subpapillary venous plexus while reactive hyperemia was induced.

The forearm, supported at heart level, was inserted into a transparent celluloid chamber through a thin rubber diaphragm which fitted the forearm snugly but did not congest it. While pressure on the skin was elevated to the desired level (20, 40, or 60 cm. H₂O) the weighted ring could be applied to, and removed from, the skin by hanging it from a rod which entered the distal end of the chamber through a flexible rubber stopper. The time of occlusion being varied, threshold and clearing times were observed through the transparent celluloid.

A typical observation is shown in Figure 6. Long rest periods were interpolated at intervals merely to avoid the discomfort which developed when the forearm was immobilized at heart level in the chamber for periods exceeding 45 minutes. Increasing the external pressure on the skin and its blood vessels increased threshold but did not change clearing time. Table III summarizes the results of three observations on different subjects. External pressures of 20, 40 and 60 cm. H₂O increased threshold by 53, 78 and 129 per cent, respectively, whereas changes in clearing time were irregular and not significant. Skin temperatures were measured throughout; changes were too small to affect threshold. It appears that visible reactive hyperemia is more difficult to elicit both when external pressure on the skin is increased

TABLE II

Effect of elevating the forearm on visible reactive hyperemia

Subject	Threshold			Clearing time		
	Forearm horizontal	Forearm elevated	Change	Forearm horizontal	Forearm elevated	Change
	sec.	sec.	per cent	sec.	sec.	per cent
J. S., III	11	21	+91	26	28	+8
J. S., III	12	20	+67	21	24	+14
L. H. S.	11	16	+45	30	23	-23
L. H. S.	11	17	+55	25	24	-4
L. H. S.	15	24	+60	22	20	-9
W. F. G.	12	20	+67	31	25	-19
W. F. G.	10	18	+80	41	26	-37
Average change, per cent			+66			-10

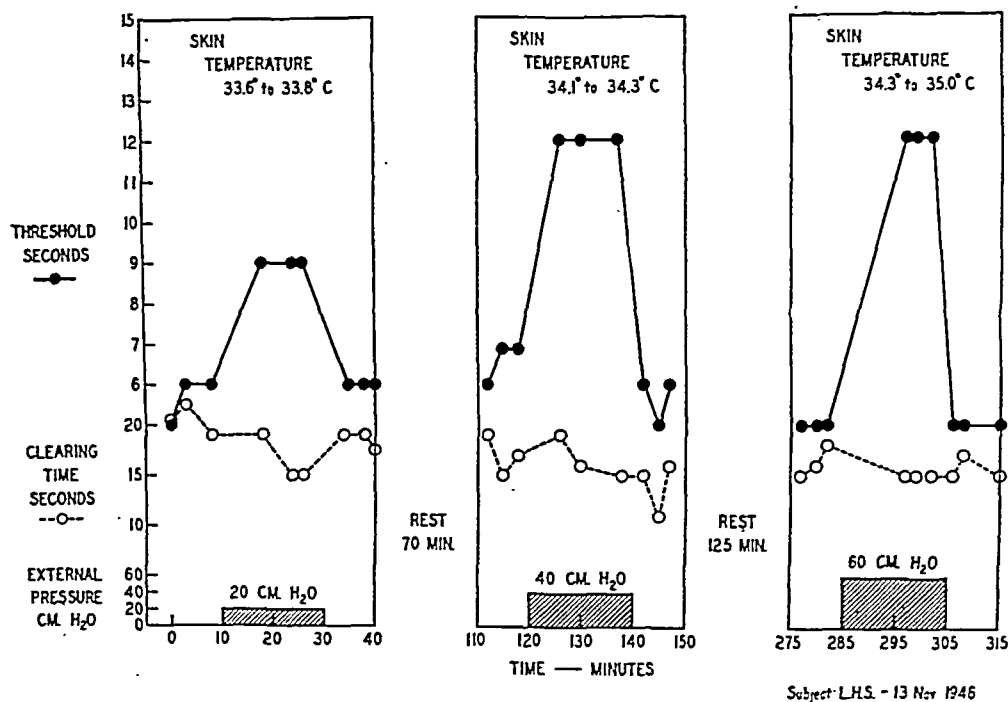


FIG. 6. CHART SHOWING EFFECT OF EXTERNAL PRESSURE ON THRESHOLD AND CLEARING TIMES

and when venous pressure is reduced somewhat by raising the forearm (Figure 5 and Table II). Decreased filling of the cutaneous subpapillary venous plexus is common to these two procedures and reduces markedly the visibility of the hyperemia which follows very brief arterial occlusion.

VI. Effect of epinephrine iontophoresis on reactive hyperemia

The disproportionately increased threshold observed from room and skin temperatures were reduced (Figure 3) suggested that vasoconstrictor tone interfered with the dilatation otherwise

produced by metabolites. This could not be concluded definitely, however, because of the concomitant reduction of metabolism in the cooled skin. To produce an increase in the tone of the minute vessels uncomplicated by other factors, epinephrine hydrochloride was introduced into the skin by iontophoresis while reactive hyperemia was tested repeatedly.

Figure 7 shows the effect of introducing 1:10,000 epinephrine hydrochloride for 10 minutes from a filter paper electrode 40 sq. cm. in area at 250 microamperes (62 microamperes per sq. cm.). Spotty blanching appeared at the anode and it was impossible to obtain a threshold reactive hyperemia even though circulation was stopped for 2 minutes, or six times the control threshold of 18 seconds. Reactive hyperemia was detected first about 11 minutes after the end of iontophoresis and then it required 2 minutes' occlusion. Clearing times were correspondingly prolonged. Reactive hyperemia returned to normal about 50 minutes after the end of iontophoresis. Results similar in every way to Figure 7 were obtained in three subjects.

In a larger series of subjects the effects of lower concentrations of epinephrine were studied

TABLE III
Effect of external pressure on reactive hyperemia

Pressure, cm. of water	Change in threshold			Change in clearing time		
	20	40	60	20	40	60
	per cent	per cent	per cent	per cent	per cent	per cent
Subject						
W. F. G.	+30	+109	+86	+26	+14	+62
L. H. S.	+50	+82	+150	-23	-4	-11
J. S., III	+80	+44	+150	+24	-12	+15
Average per cent	+53	+78	+129	+9	0	+22

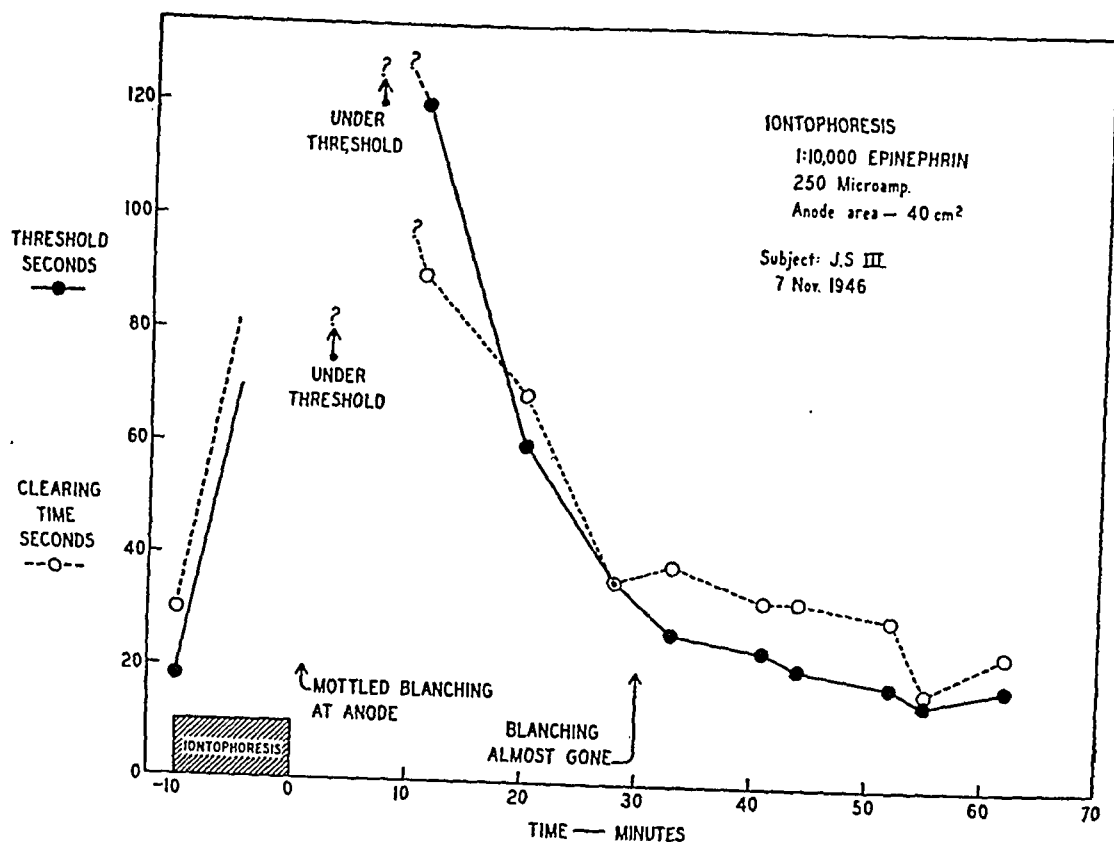


FIG. 7. CHART SHOWING INCREASE OF THRESHOLD AND CLEARING TIME PRODUCED BY PRIOR IONTOPHORESIS OF EPINEPHRINE

as shown in Table IV. Iontophoresis was here limited to 30 seconds with current strength of 36 microamperes per sq. cm.⁴ Even though the current was lower and the duration of iontophoresis was briefer, 1:10,000 epinephrine elevated threshold in all subjects by an average of over 200 per

TABLE IV
Effect of epinephrine iontophoresis using 1:10,000 to 1:1,000,000 solutions

Concentration of epinephrine	Number of subjects	Total number of experiments	Number of experiments showing increase of threshold	Average increase in threshold*	Average increase in clearing time*
1:10,000	9	19	19 (100 per cent)	259	>87†
1:50,000	11	19	16 (84 per cent)	171	>69†
1:100,000	11	21	17 (81 per cent)	92	15
1:500,000	9	12	7 (58 per cent)	54	17
1:1,000,000	5	5	1 (20 per cent)	5	0

* The per cent increase was calculated by dividing the sum of the increases by the total number of experiments. Thus with 1:1,000,000 epinephrine, one subject of the five showed a rise of 25 per cent, the others none. The average rise was 5 per cent.

† Actual value greater than recorded because procedure did not always allow time for reading full clearing time.

⁴ These observations were made by J. R. DiPalma at the Long Island College of Medicine.

cent. A significant effect was observed with concentrations down to 1:500,000. With these lower concentrations, however, considerable variability was observed in different subjects and also in the same subject from day to day, presumably owing to unequal penetration of epinephrine through the skin.

Control observations showed that the simple passage of current through the skin via electrodes soaked in distilled water or 0.9 per cent NaCl solution either had no effect on threshold or, by producing redness of the skin (13), made accurate readings impossible. Nevertheless, epinephrine in sufficient strength always produced blanching and elevated both threshold and clearing times. It seems certain that these changes were due to the constrictor action of epinephrine which reduced the ability of the minute vessels to respond normally by dilatation to the metabolites accumulated when circulation was occluded briefly.

DISCUSSION

The close relation between duration of occlusion and duration of visible redness (Figure 1) indi-

cates that the reactive hyperemia produced by local compression of the skin resembles the more generalized reactive hyperemia produced by Lewis and Grant (9) when circulation to the whole forearm was occluded by means of a pneumatic cuff on the arm. Both appear to be due to the local accumulation of metabolites but the greater sensitivity of localized reactive hyperemia made it possible to identify secondary factors which enhance or diminish the visible response.

The temperature of the skin is known to affect the degree of vasodilatation produced by arresting the circulation for a given time. Lewis and Grant (9) observed, in a limb at 36° C., that plethysmographically recognizable reactive hyperemia occurred after occlusions as brief as 5 seconds. At lower temperatures they found longer occlusions were necessary but no specific times were stated. Local reactive hyperemia responds in a similar manner to changes in temperature. Thus when skin temperature was 37° C. the average threshold (Figure 3) was only 6 seconds; at 27° C. threshold was about 30 seconds, a five-fold increase for a difference of 10° C. Average clearing time, *i.e.*, the time required for disappearance of the flush, also increased five-fold when the skin was cooled 10° C.

This change was not uniform, however, throughout the whole range of 27° to 37° C. From 37° to 32° C. the average rate of change was low, amounting to a doubling of threshold or clearing time for a lowering of skin temperature by 10° C. From 32° C. to 27° C. the average rate of change was much greater, *i.e.*, at the rate of a five- to eight-fold change for a change of 10° C. in skin temperature.

As Lewis and Grant (9) mention, the effect of tissue temperature on reactive hyperemia suggests of itself that dilatation depends on the metabolic rate of the tissue. A rise of 10° C. usually increases the rate of metabolic processes two- or three-fold. For extremities sympathectomized six months earlier, Freeman (14) found that blood flow plotted against bath temperature by the Arrhenius equation yielded a straight line. The slope of this line indicates a 2.5-fold change of blood flow for a temperature difference of 10° C. In local reactive hyperemia, threshold and clearing

time also showed changes of this order when skin temperature was above 32° C.

Below 32° C., however, the rate of change in threshold and clearing time with change of temperature became much greater. In these observations skin temperatures at the critical point, approximately 32° C., corresponded to an average air temperature of 25° C. Both these temperatures agree remarkably well with those at which Ferris *et al.* (15) observed an abrupt increase of blood flow in the hand, apparently due to a freeing of the cutaneous vessels from vasoconstrictor impulses when conservation of heat was no longer required by the thermoregulatory mechanism. On this basis it is suggested that the slow increase in threshold, as skin temperature fell from 37° to 32° C., can be explained by a slower accumulation of metabolites. The more rapid increase of threshold as skin temperature fell from 32° to 27° C. was then probably due (*a*) to continued slowing of metabolite accumulation, and in addition (*b*) to increasing vasoconstrictor tone. The latter, by resisting vasodilatation, required a larger total accumulation of metabolites, and hence a longer occlusion time or threshold to produce a visible reactive hyperemia, as well as a longer period of hyperemia, *i.e.*, clearing time, to remove those metabolites.

Venous pressure proved to be another extremely important modifying factor. When the forearm was kept at heart level, increasing venous pressure (Figure 4) reduced threshold and prolonged the clearing time. Dependency of the forearm had the same general effect. On the contrary, elevating the forearm (Figure 5) increased threshold time definitely above that observed at heart level but had little effect on clearing time. The changes were too great to explain, on the basis of change in temperature, tone of minute vessels or those small changes in blood flow that mild venous congestion or change in position might produce.

Skin color is largely determined by the amount, and the oxygenation, of the blood in the subpapillary venous plexus (12). Thus the depth of skin color of a whole extremity is less when the extremity is held above heart level than when it hangs dependent. An elevation of venous pressure can presumably affect the visibility of a given

hyperemia in at least two ways: (1) By increasing internal pressure in the subpapillary venous plexus, it may make those vessels more easily dilatable by smaller accumulations of vasodilator metabolites and hence reduce the duration of occlusion (or threshold) necessary to produce a visible hyperemia. (2) By widening passively the vessels which comprise the subpapillary venous plexus, an increased venous pressure may make a given hyperemia more easily visible because increments of intruding arterial blood remain in a given area of skin in greater amount and for a longer time and so change skin color more distinctly. Other things remaining constant both factors would not only tend to decrease threshold but also to prolong clearing time.

Elevating the forearm, by lowering venous pressure, should act in the opposite direction, *i.e.*, increase threshold because internal tension is less and because greater arterial inflow into the collapsed plexus would be required to produce a visible hyperemia. A similar prolongation of clearing time need not occur, however, because the subpapillary venous plexus tends to empty itself rapidly when the extremity is elevated. The converse also appears to hold. When the subpapillary venous plexus was collapsed by applying external pressure to the forearm, threshold rose apparently because external pressure made dilatation more difficult as well as less easily visible. Clearing time did not change, however, and the effect was similar to that observed when the forearm was simply elevated. In this connection it is noteworthy that as the forearm is progressively elevated, venous pressure does not fall to zero but to a plateau between 2 and 5 cm. H₂O (16, 17). Correspondingly, the changes in threshold produced by elevating the forearm were limited and smaller than those that could be produced by external pressure. Though clearing time was increased by venous congestion, both elevation and external pressure had no significant effect. From this it seems probable that small changes in venous pressure modified the visibility of cutaneous reactive hyperemia by mechanical effects on the subpapillary venous plexus rather than by changes of arterial blood flow.

The effect of increased tone of the minute vessels, while suggested by the studies on temperature, was demonstrated more clearly by epineph-

rine iontophoresis (Figure 7). Temperature, venous pressure and position being constant, epinephrine increased both threshold and clearing time, the former more conspicuously. These effects were very marked as long as visible blanching persisted, but in slighter degree persisted beyond the period of gross blanching.

Reactive hyperemia is obviously not a simple reaction. Though fundamentally due to the accumulation of vasodilator metabolites, the visible redness of normal skin can be affected secondarily by several other factors. Among these are (a) the temperature of the skin which determines the rate at which metabolites accumulate during an occlusion of the circulation; (b) venous pressure and position of the extremity which modify the effect of a given occlusion by mechanical effects on the subpapillary venous plexus and thereby on the visibility of a given hyperemia; and (c) the tone of the minute vessels, either of neurogenic origin, as in cold, or of local chemical origin as in epinephrine iontophoresis. Because local reactive hyperemia is affected markedly by modifying vascular tone, it can be used to detect cutaneous vasoconstriction, providing skin temperature and venous pressure in the forearm are known to be constant. The results observed with this method during exhausting exercise and other circulatory stresses are reported in another paper (6).

SUMMARY

The factors affecting cutaneous reactive hyperemia in man were studied semiquantitatively by means of a previously described method in which blood flow in the skin was stopped locally by applying a weighted ring to the volar surface of the forearm. The end points used were (a) the duration of occlusion required to produce a clearly visible ring of hyperemia (threshold) and (b) the time required for skin color to return to normal (clearing time).

Lowering of tissue temperature produced a moderate increase of threshold and clearing time between 37° and 32° C. and a very conspicuous increase of both between 32° and 27° C. Reasons are given for ascribing the former to the action of metabolites predominantly, and the latter to heightened vasoconstrictor tone acting against the dilator effect of metabolites.

Increasing venous pressure by congestion or by dependency of the forearm lowered threshold and increased clearing time. Elevation of the forearm or application of pressure to the surface of the forearm increased threshold but did not affect clearing time. It appears that these factors act by their mechanical effects on the subpapillary venous plexus and consequently on the visibility of a given increase of arterial blood flow.

Iontophoresis of epinephrine, by increasing the tone of the minute vessels, increased both threshold and clearing time conspicuously.

If venous pressure and skin temperature are kept constant local reactive hyperemia can be used to detect rapid changes in the tone of the minute vessels of the skin in man.

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THE RELATION OF SERUM BICARBONATE CONCENTRATION TO MUSCLE COMPOSITION¹

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Knowledge which enables one to predict the composition of intracellular as well as extracellular fluids is necessary for rational therapy of disturbances in body water and electrolyte. The present paper shows that, when the kidneys reach adjustment in the presence of a deficit of sodium or chloride or potassium, there is a predictable relationship between the concentration of bicarbonate in serum and the composition of rat muscle. At present, treatment of acidosis is guided chiefly by information that low concentration of bicarbonate in serum is explained by an absolute or relative deficiency of the sodium in extracellular fluids. Similarly, the therapy of alkalosis is based on knowledge that relative or absolute deficiency of chloride in extracellular fluids leads to high concentration of bicarbonate in serum. Although the changes in body electrolyte are not confined to extracellular electrolyte, knowledge of the quantitative aspects of the changes in intracellular fluids is just beginning to be developed. Darrow (1) has presented some of the known changes in composition of intracellular and extracellular fluids in a form which permits quantitative comparison of the changes in the two chief categories of body fluid and has discussed some of the implications of these quantitative relationships. The present paper expands and defines these relationships at biological equilibrium.

The experiments were designed to show the changes in composition of both serum and muscle which accompany certain types of deficit of potassium, in acidosis produced by deficit of sodium and in alkalosis produced by deficit of chloride. In each type of experiment, the analyses present the compositions after the kidneys have reached the adjustment which is attained in the presence of a deficit of one of the ions—sodium, chloride

or potassium—and are suitable to demonstrate the relation of the concentration of bicarbonate in serum to the composition of muscle.

EXPERIMENTAL METHODS

All experiments were carried out on white rats of both sexes, weighing about 300 grams. Before being used in the studies, the rats were fed Purina Fox Chow. During the experimental period three diets were used. The low potassium diet was made up as follows: Lactalbumin 18, Crisco 22, Dextrin 32, Sucrose 25, cod liver oil 1, powdered yeast 2, bone ash 2, NaCl 1 gram. In addition to the above, the normal potassium diet had 2 grams KH_2PO_4 . The rats with acidosis received the same diet without bone ash or sodium chloride. The analyses of the diets are shown in Table I.

TABLE I

Composition of diets per 100 grams

Type of diet	N	Cl	Na	K
	<i>grams</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
Low Cl	2.95	1	30	13
Low Na	2.95	1	1	1
Low K	2.95	17	34	1
Normal	2.95	17	34	13

One group of rats was adrenalectomized and kept alive for three to four days by administration of salt solution until they had recovered from the effects of the operation. Other rats were subjected to similar dietary and experimental procedures except that no operation was performed. The experimental period of three weeks was then begun by giving them the synthetic diets, but the experiments on the adrenalectomized rats were terminated in two weeks, when the rats seemed unlikely to survive.

During the experimental period the rats were subdivided into groups of four, which received daily subcutaneous injections of desoxycorticosterone acetate, desoxycorticosterone acetate in conjunction with cortical extract, cortical extract or no hormones, as is indicated in Table II. The desoxycorticosterone acetate was prepared so that about 2 mg. were given in 1 cc. of a suspension of crystals. The cortical extract was Upjohn's lipo-extract which contains 40 rat growth-survival units per cubic centimeter and is equivalent per cubic centimeter to 2

¹ Aided by a Grant from the Fluid Research Fund, Yale University School of Medicine, New Haven.

TABLE II

Group No.	Rat No.	Pe- riod	Diet*	Injec- tion	Adre- nal- ectomy	Muscle composition per 100 grams fat-free solids								Serum concentrations per liter							
						Fat	H ₂ O	N	Cl	Na	K	P	(Na) _i	H ₂ O	HCO ₃	Cl	Na	K	pH	PCO ₂	
		days				grams	grams	grams	mM	mM	mM	mM	mM	grams	mM	mM	mM	mM		mm. Hg	
1	13		N	0	0		340 6	15.3 0.3	7.1 0.5	10.0 0.6	48.9 0.6	32.4 1.5	3.1	933 5	20.9 2.4	102.6 0.6	143 2.9	5.4 0.3	7.28 0.03	44	
2	3	21	N	CE	0	3.0 0.8	343 3	14.2 0.2	5.7 0.2	8.8 0.4	46.4 0.5	31.7 0.2	3.4 0.4	935 8	22.0 1.5	108.8 2.6	138 2.7	7.2 0.6	7.23 0.03	48	
3	4	21	N	D	0	4.1 1.0	325 3	14.0 0.3	5.4 0.2	14.9 0.8	36.3 1.7	30.4 0.8	8.7 0.7	930 2	30.2 2.0	92.1 4.5	144 4.4	7.8 1.3			
4	4	21	N	DCE	0	3.0 1.1	326 8	13.2 0.4	5.7 0.3	16.6 1.4	35.5 1.4	30.1 0.9	9.8 1.3	932 4	30.0 2.4	97.4 4.7	147 1	5.0 0.2			
5	2	21	LK	0	0	11.8 1.3	314 24	15.3 0.1	5.6 0.5	8.5 0.2	39.9 0.6	31.5 0.5	6.7 0.8	928 3	30.3 1.0	94.0 5.0	147.5 1.5	5.1 0.03			
6	4	21	LK	CE	0	8.1 1.0	327 5	15.0 0.3	5.5 0.7	11.4 0.4	43.6 0.5	28.0 1.8	5.9 0.9	943 2	26.3 1.4	100.8 1.2	138 2.5	6.6 0.3	7.43 0.02	37	
7	4	21	LK	D	0	7.9 1.0	326 8	15.0 0.2	5.5 0.3	19.8 1.0	34.0 1.1	26.5 0.9	13.2 1.6	936 8	38.1 4.0	85.3 2.4	141 1.7	5.8 1.2	7.48 0.03	52	
8	4	21	LK	DCE	0	11.5 0.6	334 9	15.6 0.4	5.6 0.3	18.5 1.2	34.3 1.1	31.3 0.5	13.1 0.6	939 8	40.0 2.5	86.0 4.0	143 6	5.1 0.3	7.44 0.03	59	
9	3	14	LK	0	+	11.8	350	16.6	6.6	10.9	46.9	32.9	4.1	944 6	22.1 1.0	99.9 5.9	134 4.8	7.4 0.5	7.32 0.06	44	
10	3	14	LK	CE	+	6.5 1.9	335 8	15.6 0.8	5.7 0.3	15.9 1.0	33.5 3.3	26.6 0.5	9.4 0.9	946 3	31.6 4.0	96.4 3.9	145 2.6	4.4 1.9	7.34 0.02	60	
11	3	14	LK	D	+	11.3	331 4	15.7 0.4	4.5 0.9	20.8 2.1	34.4 2.3	30.4 0.7	15.7 2.6	943 2	39.2 1.2	88.3 5.5	139 2.9	6.1 1.5	7.40 0.08	60	
12	3	14	LK	DCE	+	6.8 1.3	338 4	15.7 0.3	6.0 0.6	20.9 2.6	33.5 1.4	30.3 1.0	13.8 1.0	943 3	38.2 2.2	93.0 3.2	143 1.4	6.5 2.5	7.43 0.03	62	
13	5	2	LNa	0	0		341 5	14.1 0.1	4.9 0.2	6.6 0.3	49.5 1.0	33.7 0.4	1.3 0.6	910 2	14.6 2.3	93.6 2.4	138.8 2.5	6.8 0.5			
14	4	6	LCI	0	0	11.0 1.0	337 6	14.7 0.1	5.0 0.4	15.2 0.6	38.2 2.0	31.7 0.8	9.5 0.6	935 1	32.8 4.0	79.5 2.1	142.7 0.5	4.3 0.6			

* N = Normal diet.
LK = Low potassium diet.

LNa = Low sodium diet.
LCI = Low chloride diet.

CE = Adrenal cortical extract.
D = Desoxycorticosterone acetate.

mg. compound E or 4 mg. of corticosterone. The daily dose of extract was 0.1 cc.

Acidosis was produced in rats by injecting into the peritoneal cavity a 5 per cent solution of glucose containing about 60 mM of NH_4Cl per liter. About 100 cc. per kilogram of rat were injected and the fluid remaining after four hours was removed through a small abdominal incision. The rats were kept on the synthetic diet without added bone ash or sodium chloride for two days. This procedure removed about 10 mM of sodium and 5 mM of chloride per kilogram of rat and at the end of the experiments the serum concentration of bicarbonate varied from 11 to 18 mM per liter. The details of the acidosis experiments are recorded in the thesis of Robert Schwartz (2).

The alkalosis experiments were reported previously as

Group 4 (3) and are represented in the present paper as Group 14. In these experiments a glucose solution containing 150 mM of NaHCO_3 per liter was injected into the peritoneal cavity and withdrawn after four hours. The rats were permitted to survive on a diet deficient only in chloride for six days.

At the end of the experimental period, the rats were anesthetized with ether, and as much blood as possible was withdrawn from the abdominal aorta by inserting a needle attached to a syringe containing mineral oil. The rats were then immediately killed by excising the heart. The muscles of the legs were removed, freed from excessive amounts of fat and nerves, and analyzed by the methods used previously in this laboratory (4). The estimation of pH was done with a glass electrode at 38° C. The determinations of pH were carried out on

serum, when sufficient blood was obtained. Unfortunately, the pH was not estimated in the later experiments, because the apparatus was out of order.

Four rats were started in each experimental group, but in some groups one rat died or blood was not obtained in sufficient quantity. Some groups are, therefore, represented by analyses of only two or three animals. Table II shows the average \pm the standard deviation. Attention is directed to the fact that the muscle analyses are calculated per 100 grams of fat-free solids. This method of presentation (3) was used because the fat-free solids are chiefly intracellular and hence the values for intracellular constituents are practically unaffected by changes in the volume of extracellular water. Furthermore, the data show no close relation of the volume of intracellular water or the concentration of intracellular electrolyte to the concentration of extracellular sodium. Hence the changes in intracellular electrolyte are most simply represented in terms of fat-free solids.

The intracellular sodium was calculated as follows:

$$\frac{(Cl) - 1}{[Cl]_e} = (H_2O)_e$$

$$(Na) - (H_2O)_e[Na]_e = (Na)_i$$

in which (Cl) and (Na) are total tissue chloride and sodium per 100 grams of fat-free solids; $(H_2O)_e$ is the extracellular water per 100 grams of fat-free solids; $(Na)_i$ is the intracellular sodium per 100 grams of fat-free solids. $[Cl]_e$ and $[Na]_e$ are the concentration of chloride and sodium in an ultrafiltrate of serum calculated from the serum concentrations and the serum water and an average Donnan factor of 0.96. In the first equation the 1 represents an average correction for non-extracellular chloride (4).

RESULTS

As shown in Group 1, the usual value for the muscle potassium of normal rats on a normal diet is 49 mM per 100 grams of fat-free solids. The standard deviation is somewhat deceiving, since Miller and Darrow (5) found that essentially normal rats may show values as low as 44 mM, but potassium in muscle probably does not go below this figure in normal rats. In adrenalectomized rats muscle potassium is 52, when the rats are fed a diet of the usual salt content (6). Therefore the two different sets of values for normal and adrenalectomized rats should be used in determining changes in muscle composition brought about by either the diet or the injections of cortical extract or desoxycorticosterone acetate.

The results in unoperated rats fed the normal diet are shown in Groups 1 through 4. The injection of cortical extract (Group 2) did not

change the muscle composition unmistakably though the concentration of chloride in serum is a little high (109 mM per liter). However, the injection of desoxycorticosterone acetate alone or in conjunction with cortical extract (Groups 3 and 4) lowered the muscle potassium to 36 mM per 100 grams of fat-free solids and increased the intracellular sodium to about 9 mM per 100 grams of fat-free solids. The decrease in muscle potassium and the increase in intracellular sodium following injections of desoxycorticosterone acetate have been previously demonstrated (5, 7 to 9). The present experiments show that the muscle changes are accompanied by an increase in concentration of bicarbonate in serum to 30 mM per liter. The rise in serum bicarbonate was missed in the previous work since this determination was omitted. However, the previous work showed a decrease in serum chloride and normal or high serum sodium. These findings almost certainly indicate that high concentration of bicarbonate in serum could have been demonstrated.

The results on unoperated rats fed a diet low in potassium are shown in Groups 5 through 8. Group 5 shows that diets low in potassium decrease muscle potassium to 40 and increase intracellular sodium to 6.7 mM per 100 grams of fat-free solids. Accompanying the changes in muscle, the concentration of bicarbonate in serum increased to 30 mM per liter and the concentration of chloride in serum decreased to 94 mM per liter. Though less marked, these changes are similar to those produced by injections of desoxycorticosterone acetate in rats fed a normal diet. The injection of cortical extract in rats fed a diet low in potassium (Group 6) was not followed by as great a lowering of muscle potassium or as great a rise in serum bicarbonate. However, the injection of desoxycorticosterone acetate alone or in conjunction with cortical extract (Groups 7 and 8) decreased muscle potassium to 34 and increased intracellular sodium to 13 mM per 100 grams of fat-free solids. The most striking change in these groups is the increase in serum bicarbonate to about 39 mM per liter and the reduction of serum chloride to about 85 mM per liter.

Groups 9 through 12 show the results in adrenalectomized rats on a diet low in potassium. If one recalls that a diet of normal potassium con-

tent is likely to produce high muscle potassium and may lead to early death, the diet low in potassium (Group 9) did produce an effect in preserving muscle composition at approximately normal concentrations. However, serum sodium is definitely low despite normal bicarbonate and chloride. The adrenalectomized rats receiving cortical extract (Group 10) were more vigorous than the untreated ones of Group 9. It is, therefore, interesting to observe that the muscle composition is more abnormal in Group 10. The potassium is as much reduced as in normal rats on a normal diet receiving desoxycorticosterone acetate (Groups 3 and 4) or the normal rats on a diet low in potassium receiving desoxycorticosterone acetate (Groups 7 and 8). Accompanying the change in muscle composition, serum bicarbonate is 32, serum chloride 96, and serum sodium 145 mM per liter. While the cortical extract did not produce completely normal functions in the adrenalectomized rats, the changes demonstrated are of the same type as was seen in normal rats on a diet low in potassium. For this reason, the dose of cortical extract is adequate to produce an effect in animals producing no cortical hormone, *i.e.*, the dose is adequate to produce an effect in adrenalectomized rats. The injections of desoxycorticosterone acetate alone or in conjunction with cortical extract (Groups 11 and 12) probably produce essentially the same effect in adrenalectomized rats as the same injections in normal rats (Groups 3, 4, 7 and 8). The higher value for intracellular sodium in Group 11 is dependent on the one aberrant intracellular sodium mentioned later. The lowering of muscle potassium and increase in serum bicarbonate are striking in these two groups.

The rats suffering from acidosis (Group 13) show changes in muscle composition which fit in with the above findings. Lowering of serum bicarbonate decreased the intracellular sodium while muscle potassium remained normal or slightly high.

The rats suffering from alkalosis produced by deficit of chloride (Group 14) resemble the rats on a diet deficient in potassium or receiving desoxycorticosterone acetate. Muscle potassium is 38 and the intracellular sodium is 9.5 mM per 100 grams of fat-free solids. The serum bicarbonate and chloride were 33 and 80 mM per liter.

The data have been analyzed for relationships between the concentration of serum sodium and the total intracellular water per 100 grams of fat-free solids and the apparent concentration of sodium plus potassium in intracellular water. Although there are striking variations in these two values, no correlation is found with the concentration of sodium in serum. In acute experiments a decrease in concentration of sodium in serum leads to an increase in intracellular water and a decrease in the concentration of univalent cations in intracellular water (4). In the present experiments in which the rats have reached a biological equilibrium no such relationship is found. The lack of correlation between the concentration of sodium in serum and the concentration of univalent cations in intracellular water is supported by findings in patients and experimental animals (10).

There are striking decreases in muscle phosphorus in Groups 6, 7 and 10, and somewhat smaller decreases in some other groups. These changes are not associated consistently with any type of change in the serum or other constituents of the muscle. Since the values for muscle phosphorus include all muscle phosphorus, studies of

TABLE III
*Statistical data on relationships of serum
and muscle compositions*

Average and standard deviation		Correlation coefficients	
(K) mM	40.22 ± 6.21	(K) (Na) _i	-0.90
(Na) _i mM	7.91 ± 4.48	(K) (HCO ₃) _i	-0.90
(HCO ₃) _i mM	28.65 ± 8.04	(K) (Cl) _i	0.47
(Cl) _i mM	94.39 ± 8.10	(Na) _i (HCO ₃) _i	0.94
		(Na) _i (Cl) _i	-0.57
		(HCO ₃) _i (Cl) _i	-0.59

(K) is total muscle potassium and (Na)_i is intracellular muscle sodium per 100 grams of fat-free solids. (HCO₃)_i and (Cl)_i are the respective concentrations per liter of serum.

Regression equations calculated from Table III

$$\begin{aligned}
 (K) &= 50.1 - 1.25 (Na)_i \pm 2.7 \\
 (Na)_i &= 34.0 - 0.65 (K) \pm 1.9 \\
 (K) &= 60.1 - 0.696 (HCO_3)_i \pm 2.7 \\
 (HCO_3)_i &= 75.6 - 1.165 (K) \pm 3.5 \\
 (K) &= 0.36 (Cl)_i + 6.0 \pm 5.5 \\
 (Cl)_i &= 0.61 (K) + 68.7 \pm 7.1 \\
 (Na)_i &= 0.52 (HCO_3)_i - 7.1 \pm 1.5 \\
 (HCO_3)_i &= 1.69 (Na)_i + 15.3 \pm 2.6 \\
 (Na)_i &= 37.7 - 0.32 (Cl)_i \pm 3.6 \\
 (Cl)_i &= 102.5 - 1.026 (Na)_i \pm 6.6 \\
 (Cl)_i &= 111.4 - 0.59 (HCO_3)_i \pm 3.4 \\
 (HCO_3)_i &= 93.9 - 0.59 (Cl)_i \pm 3.4
 \end{aligned}$$

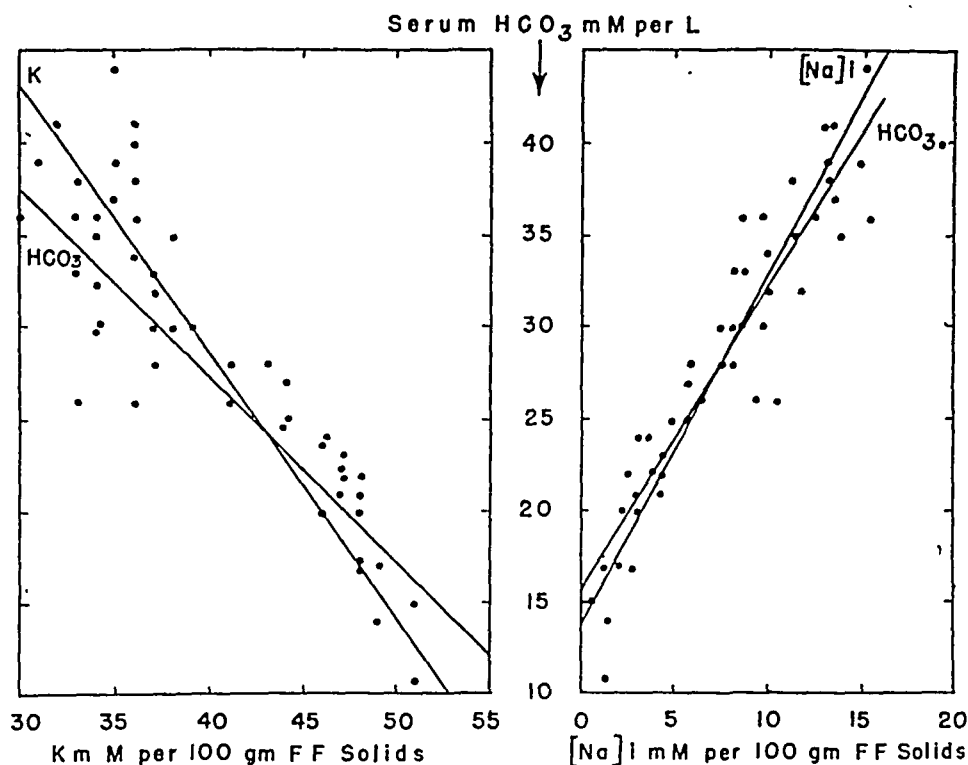


FIG. 1. RELATION OF THE CONCENTRATION OF BICARBONATE IN SERUM TO THE TOTAL POTASSIUM AND INTRACELLULAR SODIUM OF RAT MUSCLE PER 100 GRAMS OF FAT-FREE SOLIDS

the various types of compounds might throw light on the decreases in total phosphorus.

Table III shows the correlation coefficients of certain values of the serum and muscle. The chief interest is the interrelationship between serum bicarbonate, muscle potassium² and intracellular sodium which are defined in the regression equations and illustrated in Figures 1 and 2. The correlations between serum chloride and serum bicarbonate, serum chloride and muscle potassium, and serum chloride and intracellular sodium are definite, but not as precise as the correlations between serum bicarbonate and intracellular cations. For this reason the fundamental correlation of intracellular cations and serum concentrations is regarded as dependent on serum bicarbonate rather than serum chloride. The relation of muscle potassium and intracellular sodium to serum chloride is secondary relationship dependent on the inverse relationship between serum chloride and serum bicarbonate.

² It is realized that equilibrium would be theoretically more accurately stated in terms of intracellular potassium. Since the correction for extracellular potassium is small and approximately the same in all groups, the total muscle potassium was used.

As is indicated by the correlation coefficients, there are few aberrant values. In the largest deviation (one of the values in Group 11), the intracellular sodium is 19 mm. per 100 grams of fat-free solids, when the serum bicarbonate would predict a value of 14. In this case muscle potassium is essentially that predicted by serum bicarbonate. Either the determination of muscle sodium or chloride is erroneous in this case or occasional values deviate considerably from the predicted value. With this exception, the concentration of serum bicarbonate predicts the composition of muscle with respect to potassium and intracellular sodium with surprising accuracy in this series of animals. The values for the rats with acidosis fall along the lines. More values in the group with acidosis might bring out a resistance to lowering of intracellular sodium below 1 mm. per 100 grams of fat-free solids. Previous work has indicated that muscle potassium seldom rises over 50 mm. per 100 grams of fat-free solids except when serum potassium is increased (5, 7). For this reason acidosis may not lead to great increase in muscle potassium unless serum potassium rises. Doubtless other variables are involved in the relationship between intracellular

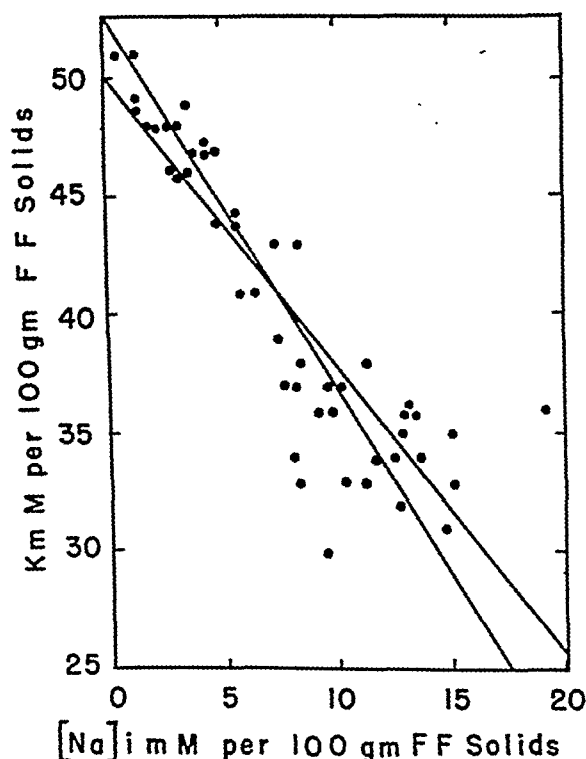


FIG. 2. RELATION OF TOTAL POTASSIUM TO INTRACELLULAR SODIUM IN RAT MUSCLE PER 100 GRAMS OF FAT-FREE SOLIDS

composition and serum concentrations, but the concentration of bicarbonate in serum is one of the most important factors.

Normal serum bicarbonate is 21 mmi. per liter and the pH is 7.28 when the blood is taken as in these experiments. Owing to the fact that the rats were anesthetized and bled from the abdominal aorta after opening the abdomen, the pH and carbon dioxide tensions are probably unreliable as measurements of undisturbed animals. It will be noticed that the carbon dioxide pressures are high in the cases with high concentration of bicarbonate in serum. Since the carbon dioxide pressure is relatively normal in the rats with normal concentrations of bicarbonate in serum, the increase in carbon dioxide pressure in the rats with high serum bicarbonate is evidence of some respiratory compensation, but not sufficient to produce the same pH as was obtained in the normal group.

Sections of the heart were examined for the lesions associated with low muscle potassium. There was no striking difference in the extent of the lesions except as associated with loss of muscle

potassium. In this respect the normal and adrenalectomized rats are alike. These observations were made because heart symptoms have seemed unduly prominent in cases of Addison's disease treated with desoxycorticosterone acetate. It was felt that cortical extract might provide some protection from the lesions produced by desoxycorticosterone acetate, but the histological sections do not support this supposition. Since the intake of sodium chloride was about the same in these experiments, this finding is in agreement with recent work which indicates that renal and heart lesions require the combined effect of sodium chloride and desoxycorticosterone acetate (11).

DISCUSSION

The experimental demonstrations of the correlation between the serum bicarbonate, muscle potassium and intracellular sodium have been obtained by four methods of producing changes either in the serum or muscle. First the diets low in potassium produce loss of potassium from the cells owing to inability of the kidneys to prevent loss of potassium when the diets contain little potassium and considerable sodium chloride. The importance of sodium chloride was brought out in unpublished observations on rats fed a diet similar to the low potassium diet of the present experiments except that no sodium chloride was added. This diet did not produce striking change in muscle composition or serum bicarbonate. Second, desoxycorticosterone acetate augments the excretion of potassium so that deficit of muscle potassium develops on normal diets. Desoxycorticosterone acetate also aggravates the effect of diets low in potassium. Third, the experiments previously published (3) produced alkalosis by injecting sodium bicarbonate in 5 per cent glucose into the peritoneal cavity. If a diet deficient in chloride was given for six days after removing the fluid equilibrated in the peritoneal cavity, the muscles lost potassium and gained intracellular sodium, and the concentration of bicarbonate in serum remained high. Fourth, acidosis produced by deficit of sodium induced the opposite effect on muscle. Thus, it seems clear that there is a biological equilibrium or biological steady state in which these three variables bear predictable relationships to each other, when renal

function is carried out in the presence of deficit of one of the ions—sodium, chloride or potassium. The biological equilibrium may be initiated either by a primary change in serum bicarbonate or muscle potassium and is the same in normal and adrenalectomized rats.

The equilibrium must be regarded as biological rather than chemical because of the relatively long time required to reach the relationships. After an interval of four hours, Hastings and Eichelberger (12) found increase in cell water, but did not measure intracellular base in their experiments on alkalosis. Yannet (13) did not find shifts of electrolyte in cats with alkalosis after an interval of about 24 hours. Flexner and others (14) found equal distribution of deuterium oxide and radioactive isotopes of sodium and potassium much more rapidly.

Whatever the mechanism for bringing about the effect of serum bicarbonate on the composition of cells, the relationships must be maintained and limited by the ability of the kidneys to regulate extracellular concentrations. The development of potassium deficit on a diet low in potassium can be explained by the fact that urine is never completely devoid of potassium. When potassium is lost from the cells, it is not difficult to understand that sodium replaces the lost cation. However, it is not easy to understand why this change is accompanied by high bicarbonate and low chloride in the serum. It would seem that, if the maintenance of extracellular concentrations were the chief renal function, the kidneys could maintain normal extracellular concentrations. The effect of desoxycorticosterone acetate is brought about by a change in the excretion of sodium and potassium by the kidneys and the end result is analogous to the effect of diets low in potassium. When high serum bicarbonate was produced by deficit of chloride, it is clear that extracellular concentrations and volumes cannot both be restored without replacement of chloride, but it is not easy to explain why these changes in extracellular concentrations lead to loss of potassium and retention of sodium in the cells. Since cell membranes are considered permeable to sodium and potassium, high bicarbonate in serum must either facilitate the transfer of potassium out of the cells or increase the entrance of sodium. The other possible explanation would be that alkalosis increases the rate

of potassium excretion. This explanation is unlikely, since the change in the cells takes place in the presence of abundant potassium in the diet. We are forced to conclude that the three variables are dependent and tend to adjust one to another so as to preserve the relationships exhibited in the regression equations and Figures 1 and 2.

There is considerable evidence that this biological equilibrium is exhibited by patients. In the cases of Cushing's syndrome with high serum bicarbonate, low chloride and low potassium (15, 16), the alkalosis responded to potassium chloride but not sodium chloride or ammonium chloride. Power and Kepler (17) found low potassium and high sodium in muscle taken at biopsy from one of these cases. Furthermore, the analogy with the effects of desoxycorticosterone acetate is supported by the fact that the alkalosis disappeared when an adrenal cortical tumor was removed and recurred when metastatic growths developed. The present data provide an explanation of the persistent alkalosis in cases of Cushing's syndrome-developing deficit of potassium.

Observations have shown that the alkalosis of vomiting is sometimes accompanied by low concentration of potassium in serum (18). One of the authors has observed one case of gastric tetany which persisted despite administration of abundant amounts of physiological saline. He also has observed a balance of sodium, potassium and chloride during recovery from dehydration following vomiting that indicated the presence of a large amount of intracellular sodium and a deficit of potassium. If these evidences of changes in cell composition in gastric alkalosis can be confirmed, gastric alkalosis must be considered to lead to deficit of potassium and excessive retention of sodium in the cells, and treatment of these disturbances with potassium chloride as well as sodium chloride is likely to prove as advantageous as the introduction of potassium chloride in the treatment of diarrhea (19, 20).

The observation of persistent high serum bicarbonate in certain lung diseases is also probably accompanied by the characteristic changes in the cells. Darrow and Sarason found high intracellular sodium and low potassium in rats subjected to low atmospheric pressure for seven days (21). Although the authors did not attribute the change in

muscle composition to alkalosis, nor measure the serum bicarbonate, it is likely that compensated respiratory alkalosis explains the change.

The first evidence of the dependence of tissue composition on serum bicarbonate was recognized in a case of "congenital alkalosis with diarrhea" (22). In this patient the stools were always voluminous and watery and contained more chloride than sodium. As the boy developed more alkalosis, potassium in excess of nitrogen was lost in the stools or urine, and sodium was retained in the cells. During recovery from profound alkalosis, administration of potassium chloride facilitated partial restoration of serum bicarbonate; the retentions of electrolyte indicated that large amounts of sodium left the cells as potassium was retained, but potassium and sodium chloride were unable entirely to overcome the alkalosis. The author felt that the excessive loss of chloride in the stools was the fundamental difficulty. In studying a similar case, Gamble and others (23) postulated that there might be a fundamental difficulty in which the body adjusted preferentially to alkalosis. The present study makes this hypothesis tenable, if one could prove that the muscles and perhaps all tissues preferentially contained excessive amounts of intracellular sodium. Such a disturbance in intracellular composition is more difficult for one of the authors to visualize than a peculiar defect in intestinal absorption leading to alkalosis. In any case, the demonstration of the dependent relationship between alkalosis and muscle composition explains the excretion of potassium in the urine despite a deficit of muscle potassium. It also explains the therapeutic failure of acid salts and the refractoriness of the cases to all forms of treatment.

It must be kept in mind that the relationships under discussion may be expected to be found only when renal function has made the appropriate adjustment. Thus, we know that there are variations in one or more of the variables without the predicted changes in the others, when there is dehydration. One may be permitted to call these types of disorders unstable states with respect to the relationship between the composition of intracellular and extracellular fluids. For instance, the studies on diarrhea (19) show that this type of acidosis and dehydration is practically always accompanied by a considerable deficit of potassium,

but intracellular sodium may be low or high. The regression equations predict high muscle potassium and low intracellular sodium in acidosis. The low intracellular sodium is contrary to the expectation of high intracellular sodium accompanying loss of muscle potassium. The high intracellular sodium is predicted by the potassium loss, but is contrary to the prediction from low serum bicarbonate. Diabetic coma (24, 25, 26) probably shows similar changes in the cells accompanying acidosis and in this sense also exhibits an unstable state in the relationship between extracellular fluids and tissue composition.

One may speculate that the lack of a relatively stable equilibrium in these usually dependent variables explains some of the findings in these cases. It is obvious that deficit of potassium explains the large doses of sodium bicarbonate necessary to overcome acidosis if sodium chloride and sodium bicarbonate are administered without potassium. It is not so clear how acidosis and dehydration rapidly become manifest in some cases. The authors have in mind babies with diarrhea who suddenly manifest marked symptoms of acidosis and dehydration despite no increase in watery stools. In these cases, if deficit of potassium develops gradually and intracellular sodium remains low or normal, an unstable state develops. Under these circumstances, acidosis and dehydration of extracellular fluids could suddenly be aggravated if sodium were rapidly transferred to the cells in consequence of the unstable state. If the transfer of sodium is equivalent to 5 per cent of the intracellular potassium, the decrease in extracellular sodium would be equivalent to one-half of the normal extracellular bicarbonate or one-tenth of the normal extracellular sodium. Since the extracellular fluids are already depleted in a patient with diarrhea, a very small shift in sodium would produce a marked aggravation of extracellular dehydration and acidosis.

On the other hand, the relationship of muscle composition to serum bicarbonate must be considered in treating disturbances in body electrolyte from the point of view of the ultimate equilibrium dependent on the tissue deficit. In diarrhea and probably also in diabetic coma (24, 25, 26), if restoration of serum bicarbonate becomes the chief criterion of successful treatment, biological equilibrium will not be obtained with a normal serum

bicarbonate, but with a high serum bicarbonate if deficit of potassium is not restored. For instance, a rat with a muscle potassium of 30 mM per 100 grams of fat-free solids (about two-thirds of normal figure) is in a biological state of equilibrium when serum bicarbonate is 40 mM per liter and intracellular sodium is 14 mM per 100 grams of fat-free solids. If a baby behaves like a rat in this respect, the sodium in the muscle cells would be about 14 mM per kilogram of body weight. This amount of intracellular sodium would be about three times the normal value and would be equivalent to over twice the normal amount of bicarbonate in extracellular fluids. Looking at the state of equilibrium from the point of view of the serum, profound alkalosis would be produced if administration of sodium chloride and sodium bicarbonate were forced until the kidneys reached biological equilibrium in the presence of potassium deficit. Alkalotic tetany is, therefore, not the result of a failure of renal function when there is deficit of muscle potassium, but alkalosis may be anticipated as the actual result which the kidneys tend to maintain. Since alkalosis often leads to low serum calcium, high concentration of serum bicarbonate may precipitate low calcium tetany.

Some such course of events probably explains the high incidence of tetany reported by Rapoport and others (27) in babies treated by prolonged intravenous therapy with solutions of glucose, sodium chloride and sodium bicarbonate. Since administration of calcium chloride would be effective in low calcium tetany and would tend to produce acidosis, the favorable effects of calcium salts when used in conjunction with sodium chloride and sodium bicarbonate are readily explained (27, 28). In contrast to their results, however, the authors have seldom observed low calcium tetany and have not encountered alkalosis when using potassium chloride as well as sodium chloride and sodium bicarbonate in treating infantile diarrhea.

The relationship of serum concentrations to muscle composition are also relevant to the practice of using the concentration of chloride in serum as an index of therapy. If deficit of potassium is present, serum chloride tends to be low at biological equilibrium. Administration of sodium chloride will not overcome the abnormality in serum and will probably aggravate the deficit of potassium in the muscles. In the treatment of Ad-

dison's disease with desoxycorticosterone acetate, low serum chloride may indicate that too large rather than too small a dose of the drug is being used.

As in previous studies on rats, the serum potassium bears no precise relation to muscle potassium. In part, this finding may be dependent on changes in the level of serum potassium accompanying anesthesia. However, high serum potassium has been the rule when treatment is begun in patients suffering from dehydration due to diarrhea (19) and in diabetic coma (26). Robinson (29) and Largaia and Vidal (30) found that the concentration of potassium was frequently low in babies with intestinal intoxication. During treatment, the potassium concentration often reached extremely low values and if there was no tendency to return to normal, such patients died (29). Although many conditions accompanied by deficit of body potassium show low serum potassium, the exceptions are so many that the level of serum potassium cannot be relied upon as an index of the state of intracellular potassium. Nevertheless, the authors do not believe the potassium concentrations of this study should be accepted without reservations, since rats are unsuitable animals for demonstrating the relation of the concentration of potassium in serum to muscle composition owing to the difficulty in obtaining blood under resting conditions. Clinical studies must be made to evaluate the value of serum potassium as an index of deficit of potassium in tissues in various conditions.

SUMMARY

The composition of serum and muscle was determined in normal and adrenalectomized rats subjected to conditions leading to adjustment of body electrolyte in the presence of a deficit of one of the ions—sodium, chloride or potassium. Deficit of potassium was maintained by a diet low in potassium or in injections of desoxycorticosterone acetate. Deficit of sodium was produced by injecting NH_4Cl in glucose into the peritoneal cavity and withdrawing the fluid, and maintaining the rats on a diet deficient in sodium. Deficit of chloride was produced by injecting a solution of NaHCO_3 in glucose into the peritoneal cavity and withdrawing the fluid, and maintaining the rats on a diet deficient in chloride.

The data demonstrate a high degree of correlation between the concentration of bicarbonate in serum, muscle potassium and intracellular sodium. Thus, serum bicarbonate varies directly with intracellular sodium and inversely with muscle potassium. Muscle potassium and intracellular sodium show inverse relationships. These relationships may be considered a biological equilibrium which is attained when renal adjustment is made in the presence of a deficit of sodium or chloride or potassium.

Adrenalectomized rats show the same relationships as normal rats.

The therapeutic implications of these findings are briefly discussed. First, the relationship is known not to be maintained in dehydration and in such states we may term the lack of relationship an unstable state with respect to the relationship between extracellular and intracellular fluids. Second, patients with dehydration accompanied by deficit of potassium may be expected to develop alkalosis when they are treated solely with sodium and chloride. Third, alkalosis may be expected to be relatively refractory to treatment with sodium chloride, if a deficit of potassium persists. Fourth, the dose of sodium bicarbonate necessary to overcome low serum bicarbonate is always uncertain, since sodium may enter or leave the cells depending on the presence or absence of an excess of intracellular sodium and the presence or absence of a deficit of potassium.

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ASSOCIATION ANNOUNCEMENT

The 40th annual meeting of the American Society for Clinical Investigation will be held at the Chalfonte-Haddon Hall, Atlantic City, N. J., on Monday, May 3, 1948, at 9 a.m.

For those who may be interested, the annual meeting of the American Association for Research in Psychosomatic Problems will be held at the same hotel on Saturday, May 1, at 9 a.m., and

The annual meeting of the Association of American Physicians will be held on Tuesday and Wednesday, May 4 and 5, also at the Chalfonte-Haddon Hall.

THE INFLUENCE OF CLOTHING, WORK, AND AIR MOVEMENT ON THE THERMAL EXCHANGES OF ACCLIMATIZED MEN IN VARIOUS HOT ENVIRONMENTS

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During the war the need for the study of the mechanism of heat loss at high temperatures became evident. Although extensive calorimetric studies of man's thermal exchanges under certain environmental conditions have been made, quantitative calorimetric data on men in very hot environments have been missing (1, 2). Technical difficulties essentially preclude the possibility of using direct calorimetry to secure such information. However, the *partitional* calorimetric approach of the Pierce Laboratory (3) does allow a quantitative description of thermal exchanges in these hot environments which hitherto were considered largely in terms of qualitative patterns. In the present work, such a *partitional* calorimetric study was made of men in the heat to extend the measurements of environmental load factors and to describe man's responses to high environmental temperatures. This communication gives the basic calorimetric and physiological data obtained and indicates the effects of clothing, work, and air movement on the routes of thermal interchange in a series of hot environments. The data, although not as exact as may be desired, may therefore be useful.

PROCEDURE

Partitional calorimetric studies were made on four well-acclimatized, healthy young male subjects, whose ages ranged from 20 to 22 years; heights, from 166 to 185 cm.; weights, from 62 to 74 kgm.; and surface areas from 1.7 to 1.9 m². Acclimatization was achieved by having the subjects, dressed in herringbone twill uniforms, walk daily for a period of four hours in an environment having dry bulb temperatures (D.B.) of 120° F. and wet bulb temperatures (W.B.) of 88° F.

Following acclimatization, calorimetric investigations were made on these men. The fourth, or reserve subject replaced subject no. 3 after the eleventh day of the test.

The tests were conducted in a laboratory hot room within a black sheet metal wind tunnel which measured 5½ ft. by 7½ ft. by 20 ft. The dry and wet bulb temperatures were maintained to within 1° F. of the stated values. Six 24-inch fans at the discharge end of the tunnel produced linear air flow, controlled by louvers and/or fan speed. Eight-inch galvanized pipes served as air straighteners at the inflow end. The men stood or walked on the treadmill which constituted the central portion of the tunnel floor. A speed of 3 m.p.h. and a grade of 3.0 per cent were employed in the walking tests, resulting in an average energy expenditure of 160 Cal./m²/hr. Energy expenditures during standing averaged 55 Cal./m²/hr.

The seven environments and the five wind velocities employed are given in Table I. Each of the three men was studied standing nude, standing clothed, and walking clothed once in each of the 35 hot environmental conditions. Exposure to the environments was randomized so that severe and less severe stress periods were well interspersed with each other. The test periods were 30 minutes long. Difficulties in preventing loss of unevaporated sweat precluded experiments on the nude walking man. Throughout the study, observations were repeated in a "base" environment (D.B. 120° F., W.B. 88° F., wind velocity 300 ft./min.) on days 1, 10, 20, 29, 38, and 47, to obtain information regarding the constancy of response of the subjects to a given environment (Figure 1).

The men spent 7½ hours in the heat each day except Sunday. In the morning, after marching for 70 minutes in the hot room for equilibration, the test run was made

TABLE I
Environments in which men were studied

	Dry bulb temp.	Wet bulb temp.	Relative humidity	Wind velocity
	degrees F.	degrees F.	per cent	feet/min. (approx.)
1	90	70	37	30, 75, 150, 300, 600
2	96	72	31	30, 75, 150, 300, 600
3	96	83	58	30, 75, 150, 300, 600
4	96	91	83	30, 75, 150, 300, 600
5	105	74	23	30, 75, 150, 300, 600
6	120	78	12	30, 75, 150, 300, 600
7	120	88	28	30, 75, 150, 300, 600

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³ Industrial Hygiene Foundation of America, Pittsburgh, Penna.

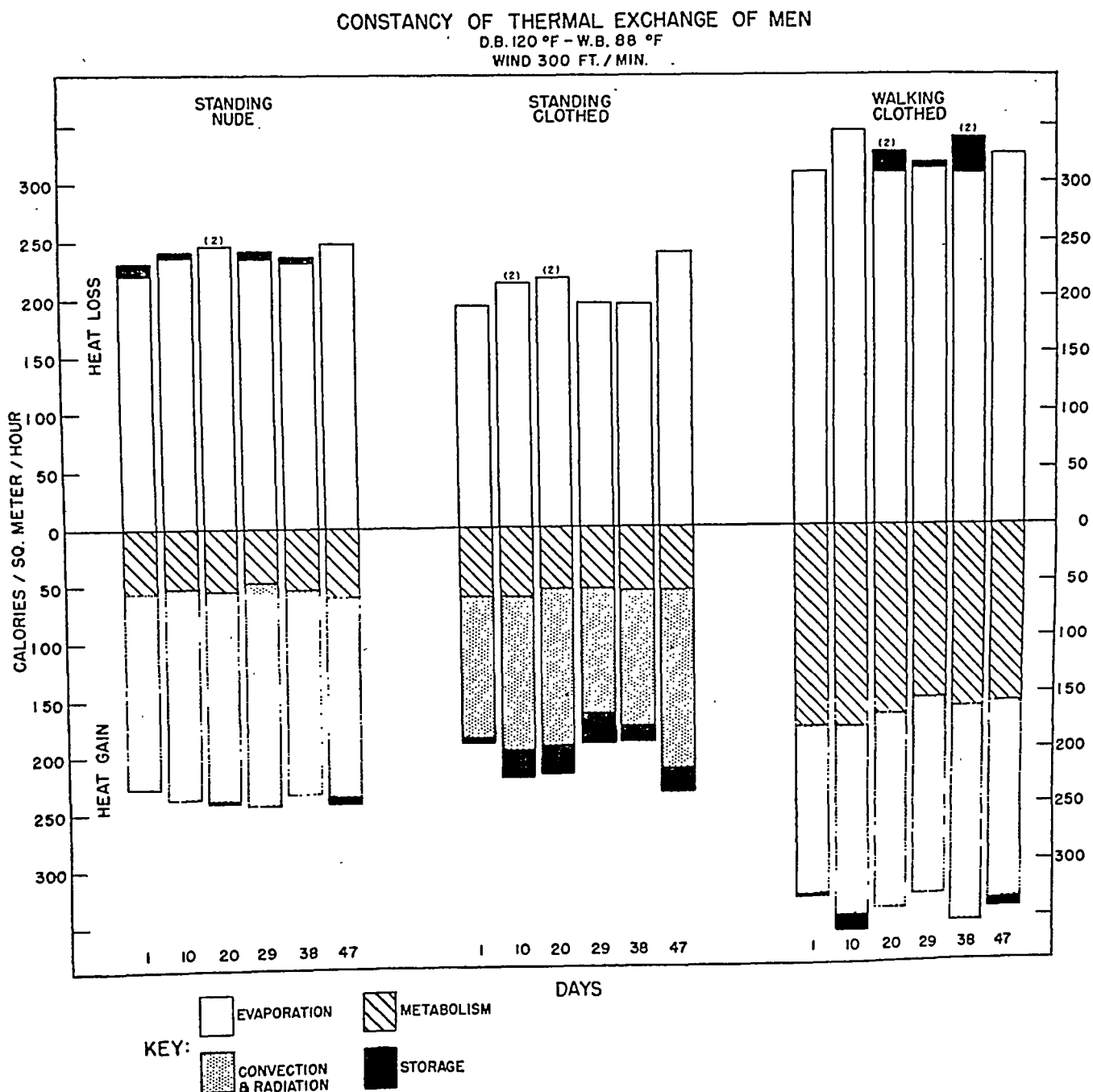


FIG. 1. CONSTANCY OF THERMAL EXCHANGES OF MEN STUDIED PERIODICALLY DURING TWO MONTHS, WITH A COMPARISON OF THE NUDE, CLOTHED AND WORK STATE

All studies carried out at D.B. 120° F.—W.B. 88° F. in a wind of 300 ft./min. Data are average of three men.

on the walking man. In the afternoon, experiments were done on the men standing nude or clothed. Each of these tests was preceded by a special equilibrating stand of 20 minutes.

During the clothed experiments the subjects wore well-laundered two-piece herringbone twill fatigue uniforms, light wollen socks, cotton shorts and field shoes. A completely dry outfit was donned prior to the start of the test period. Sweat losses from drippage were found to be insignificant. In nude trials, the men stood over shallow trays containing mineral oil to collect un-

evaporated sweat. Water salted to 0.1 per cent was given in amounts approximating sweat loss just before the test period, and at midpoint in the walking experiments.

Table II presents the types of data collected during each experiment. A detailed account of the procedures, theoretical basis for and treatment of those data has been published (4). Briefly, the collected data are converted into quantitative expressions of thermal exchange by utilization of the basic thermal exchange equation: $M + E \pm S \pm C \pm R = 0$ where the symbol

\dot{M} = rate of metabolic heat production,
 \dot{S} = rate of change in body heat content or storage,
 \dot{E} = rate of evaporative heat loss,
 \dot{C} = rate of thermal exchange by convection, and
 \dot{R} = rate of thermal exchange by radiation.

\dot{M} and \dot{E} were measured directly, \dot{S} was calculated from the skin and rectal temperature changes, while $\dot{C} + \dot{R}$ were determined by difference as defined in the above equation. No attempt has been made in this overall treatment to separate convective and radiant heat exchange; one method for such separation has been applied to these data (4). All figures are expressed as Cal./m²/hr.

RESULTS AND DISCUSSION

Table III affords a survey of the average responses of the three men under differing conditions of activity and environment. Only the extremes of wind velocity are represented in the table, but it can be seen from Figures 2 to 4 that the intermediate data are in an orderly sequence. At any given air velocity, increasing the work, in-

TABLE II

Experimental data collected during each test period

Data	Method	Frequency
Environment:		
1. Dry bulb temperature (at 1- and 6-foot levels)	Psychrometer	3
2. Wet bulb temperature (at 1- and 6-foot levels)	Psychrometer	3
3. Wall temperature (6 presenting tunnel surface)	Radiometer	2
4. Air velocity	Hot wire anemometer and anor velometer	3
Subject:		
1. Rectal temperature	Clinical thermometer	2
*2. Skin temperature	Radiometer and thermocouple	3
*3. Clothing (surface) temperature	Radiometer	3
4. Oxygen consumption	Open and closed circuit systems	2
5. Heart rate	Palpation	3
†6. Evaporative sweat loss	Weighing	2
‡7. Total sweat loss	Weighing	2

* Integration of six areas, viz., Cheek, Chest, Back or Calf, Palm, Thigh and Upper Arm.

† Total sweat loss minus weight of sweat dripped and towed off body.

‡ Change in weight of nude subject with correction for water consumed and excess weight of CO₂ excreted over O₂ consumed.

creasing the heat load or wearing clothing led to elevations in the sweating rate, heart rate, rectal and skin temperatures.

Analysis of Table III reveals that in the conditions studied, increasing the wind velocity from 30 to 600 feet per minute did not produce any striking change in the rectal temperature. In no case was the average rectal temperature changed 1° F. However, such an increase in air temperature was generally associated with significant reductions in the skin temperatures of the subjects, whether nude or clothed, standing or marching. Surface temperatures in clothed men followed this pattern of the skin temperatures at environments in which the temperature did not exceed 96° F. In the 105° and 120° environments the surface temperature rose with increasing air movement, occasionally attaining values above 105° F. Total sweat loss was most strikingly reduced by an increase in wind velocity in the tests at 120° on clothed subjects. Appreciable pulse rate reductions occurred with an increase in air velocity, in clothed men working at wet bulb temperatures of 88° or 91°.

The results of the partitioned calorimetric analysis are given in block form in Figures 1 to 4. The ordinate represents heat exchange in Cal./m²/hr.; heat loss being represented above and heat gain below the dividing line. Along the abscissa the environments are presented, with each group of blocks showing the effect of five progressively increasing air velocities for a given dry and wet bulb temperature. The results on nude, clothed and working men are presented in individual charts. The data are the averages of three men except where the numeral (2) appears indicating that the averages are for two men.

Figure 1 shows the degree of constancy in the physiological thermoregulatory mechanisms, since it presents data on men reexposed to exactly the same environment on six different days over a two-month period. These data serve as an aid in evaluation of the significance of changes seen in the other charts.

Metabolic heat production for a given amount of work remains unchanged irrespective of change in environmental conditions. Convective and ra-

TABLE III

Effect of environment, activity and clothing on physiological responses of acclimatized men
Average data on three men during half-hour exposure

Environment	Wind velocity	Activity	Clothing	Rectal temperature		Skin temperature		Surface temperature		Sweat		Pulse rate (minutes)	
				Final	Change	Final	Change	Final	Change	Total	Evaporation	Final	Change
	<i>ft./min.</i>									<i>gm./hr.</i>	<i>per cent</i>		
D. B. 90° F.	30	Standing	0	98.9	0.2	93.3	-1.0	—	—	158	98	82	1
		Standing	+	99.2	0.3	93.6	-0.7	94.8	-0.2	208	66	90	2
		Walking	+	99.4	-0.1	93.0	-0.9	93.6	-0.8	603	60	106	4
W. B. 70° F.	600	Standing	0	98.8	0.2	92.0	-1.4	—	—	152	100	79	-2
		Standing	+	99.0	0.0	91.4	-1.1	93.6	0.2	183	88	85	0
		Walking	+	99.2	-0.1	90.8	-1.2	91.6	-0.9	564	73	97	-4
D. B. 96° F.	30	Standing	0	99.0	0.3	93.8	-1.1	—	—	262	94	75	-2
		Standing	+	99.1	0.3	93.7	-2.0	96.8	-1.6	395	56	84	3
		Walking	+	99.3	-0.1	92.1	-2.4	93.8	-1.8	1019	50	99	0
W. B. 72° F.	600	Standing	0	99.4	0.3	93.4	-1.3	—	—	279	99	103	9
		Standing	+	99.6	0.4	92.7	-1.7	96.0	-0.5	278	86	103	2
		Walking	+	99.6	-0.2	92.1	-1.2	94.6	-0.8	670	74	105	3
D. B. 96° F.	30	Standing	0	99.4	0.3	94.6	-0.6	—	—	316	70	93	1
		Standing	+	99.7	0.4	95.2	-0.8	95.6	-1.5	815	24	100	4
		Walking	+	100.1	0.4	95.6	-0.5	96.0	0.0	1666	26	108	-3
W. B. 83° F.	600	Standing	0	99.0	0.2	93.0	-0.3	—	—	292	100	82	7
		Standing	+	99.0	0.2	93.2	-2.3	94.9	-0.3	433	69	73	-10
		Walking	+	99.5	0.1	93.8	-2.8	92.9	-1.3	1397	49	103	10
D. B. 96° F.	30	Standing	0	99.7	0.3	96.5	-0.1	—	—	815	17	97	7
		Standing	+	99.9	0.3	96.3	0.4	96.7	0.1	1270	12	102	12
		Walking	+	102.4	1.6	98.6	0.2	98.5	-0.5	2357	13	159	18
W. B. 91° F.	600	Standing	0	99.6	0.2	94.1	-0.3	—	—	351	77	102	2
		Standing	+	99.7	0.3	94.6	-1.6	94.0	-1.2	814	34	106	-4
		Walking	+	101.7	0.8	97.3	-0.4	95.9	-0.7	2275	24	137	15
D. B. 105° F.	30	Standing	0	99.7	0.7	93.4	-1.0	—	—	388	92	91	2
		Standing	+	99.7	0.5	93.5	-3.9	97.6	-4.2	684	41	95	1
		Walking	+	99.4	-0.1	94.1	-2.0	95.7	-3.4	1179	47	104	-7
W. B. 74° F.	600	Standing	0	99.3	0.4	92.8	-1.7	—	—	534	99	84	3
		Standing	+	99.1	0.3	91.9	-3.6	101.3	0.9	478	78	81	-3
		Walking	+	98.9	0.2	93.3	-2.4	96.8	-1.0	1027	72	102	-2
D. B. 120° F.	30	Standing	0	99.3	0.3	95.9	-1.3	—	—	573	86	90	0
		Standing	+	99.5	0.4	96.3	-2.7	100.6	-6.1	1379	28	102	6
		Walking	+	99.8	0.4	96.6	-2.2	99.9	-4.5	1883	37	111	-3
W. B. 78° F.	600	Standing	0	100.1	0.7	95.1	-0.4	—	—	815	99	115	9
		Standing	+	100.0	0.3	94.0	-5.3	107.3	-1.7	850	71	107	1
		Walking	+	99.8	0.1	95.3	-4.1	104.5	-0.9	1345	69	106	-11
D. B. 120° F.	30	Standing	0	100.4	0.6	98.7	0.3	—	—	1351	27	125	9
		Standing	+	101.1	1.0	98.6	-1.6	100.6	-2.2	2000	18	142	16
		Walking	+	102.2	1.7	100.0	-0.2	100.6	-2.4	2650	25	171	32
W. B. 88° F.	600	Standing	0	100.2	0.4	96.6	-0.7	—	—	884	97	111	3
		Standing	+	100.2	0.5	96.7	-4.0	105.2	-1.5	1197	57	115	4
		Walking	+	101.5	1.0	97.9	-0.9	101.9	-2.3	2046	52	134	14

Data on intermediate wind velocities not given.

diant heat gain, and the compensatory evaporative heat loss in the warmer environments show a progressive increase as the air movement is increased.

The equilibration period preceding the test period served in most instances to keep storage heat at a low level. Clothing on resting men resulted

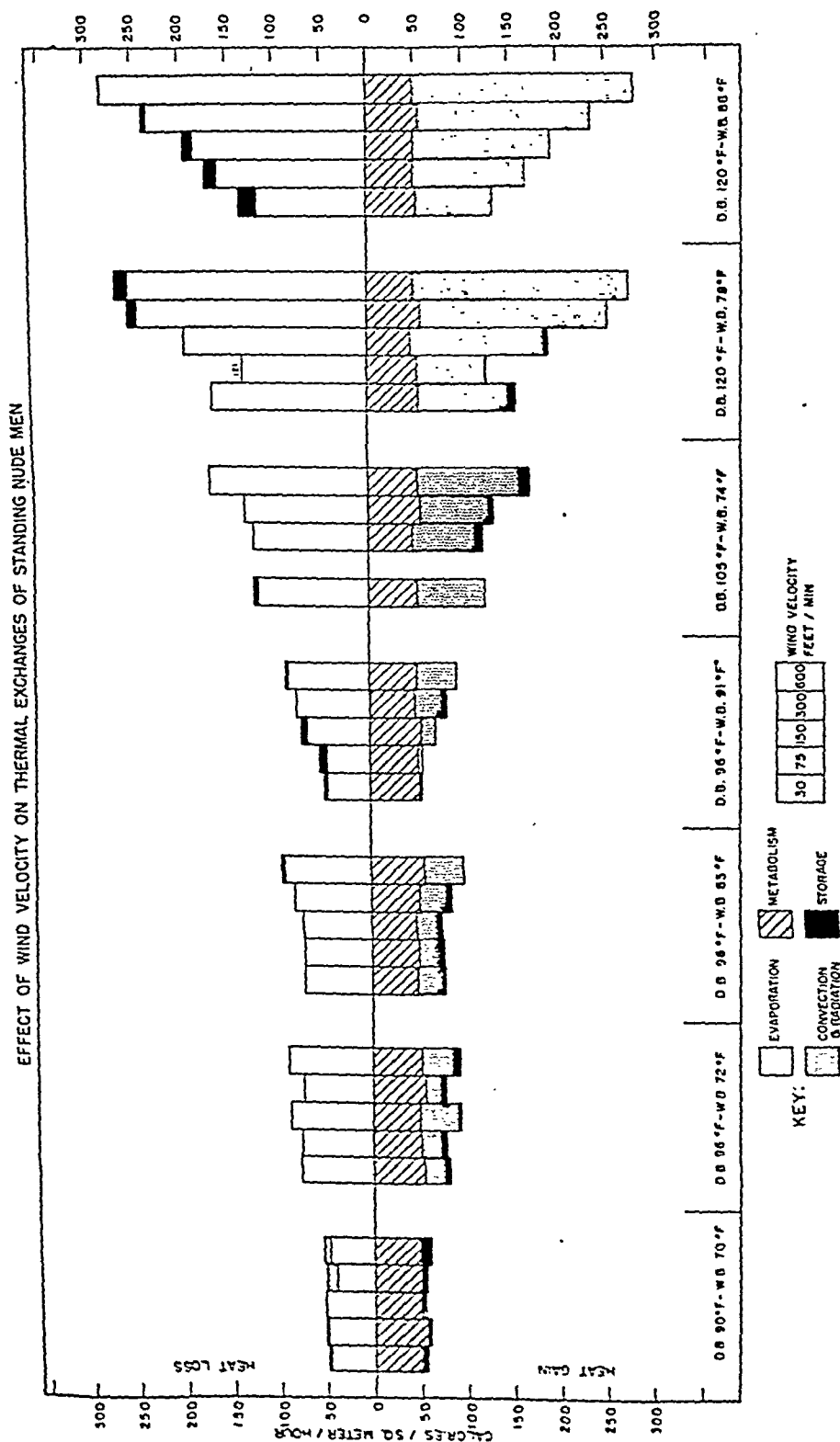


FIG. 2. EFFECT OF WIND VELOCITY ON THERMAL EXCHANGES OF STANDING NUDE MEN
Data represent average on three men in seven environments with five wind velocities each.

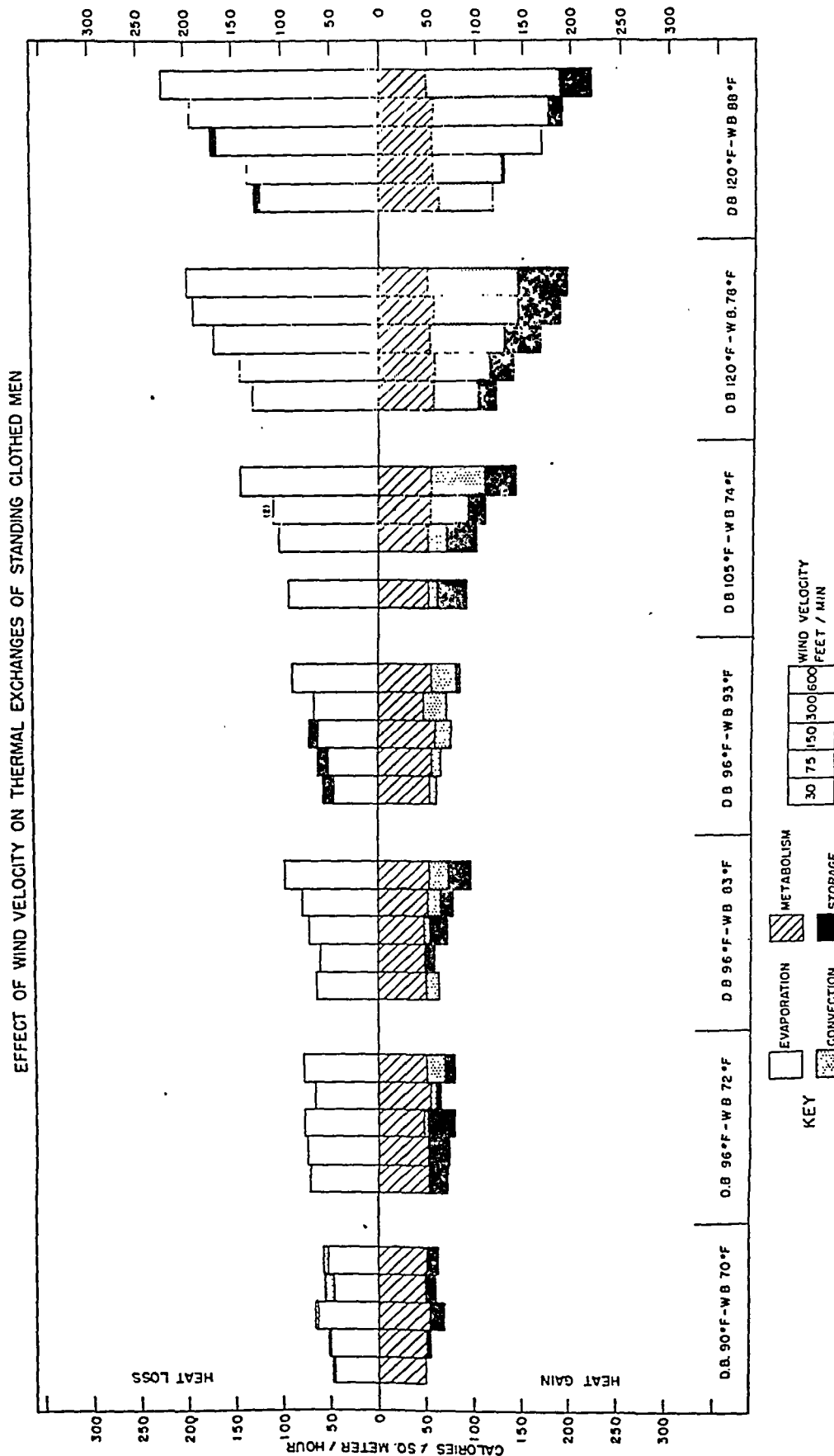


FIG. 3. EFFECT OF WIND VELOCITY ON THERMAL EXCHANGES OF STANDING MEN CLOTHED IN HERRINGBONE TWILL UNIFORMS
Data represent average on three men in seven environments with five wind velocities each.

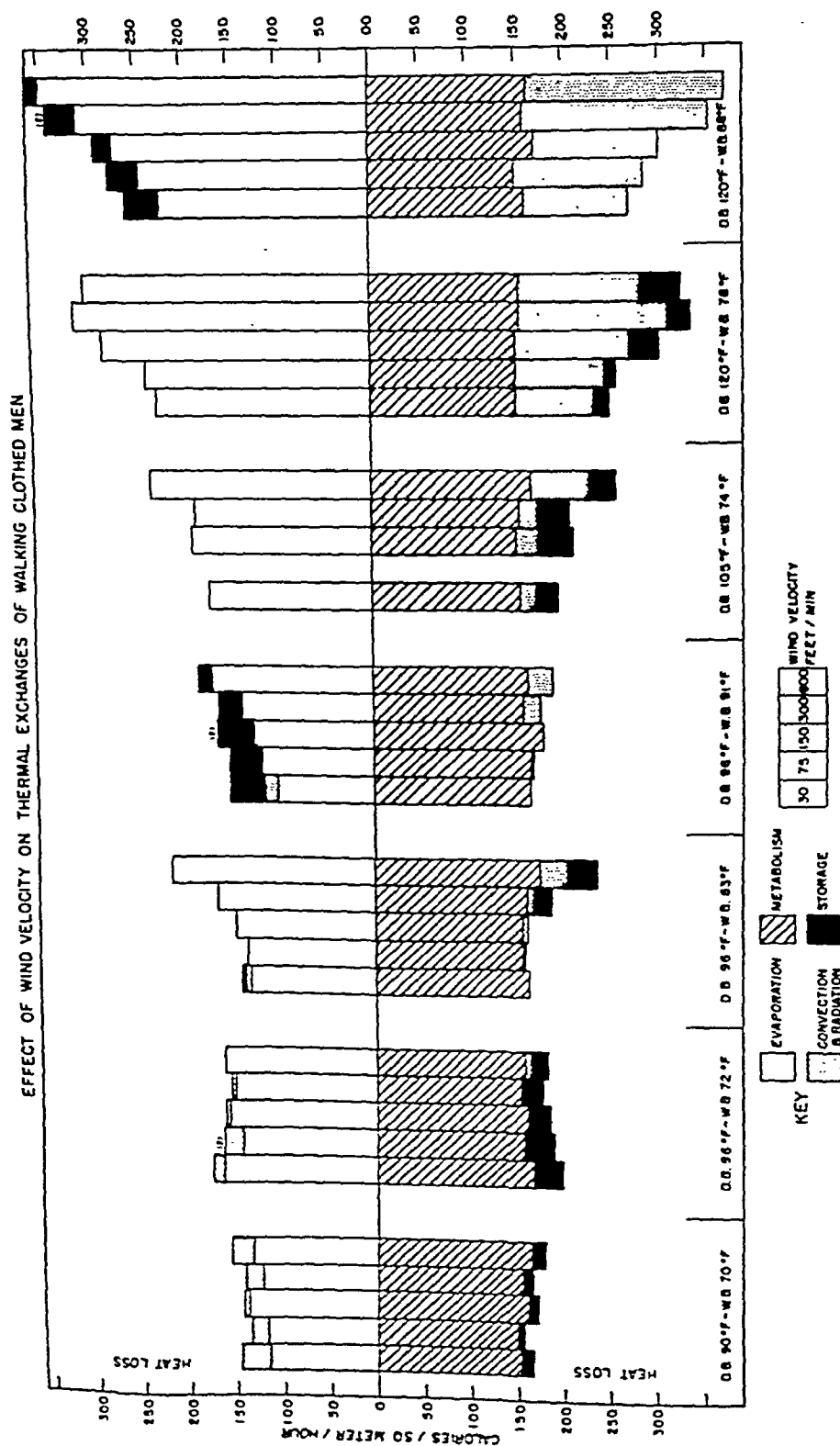


FIG. 4. EFFECT OF WIND VELOCITY ON THERMAL EXCHANGES OF MEN WALKING 3 M.P.H. ON 3% GRADE WHILE CLOTHED IN HERRINGBONE TWILL UNIFORMS

Data represent average on three men in seven environments with five wind velocities each.

NOTE: Due to an error in preparation "Heat Loss" evaluations in the lower half of the figure represent "Heat Gain" rather than "Heat Loss" as mistakenly indicated.

in smaller heat gains by convection and radiation and consequently in a smaller evaporative heat loss.

SUMMARY

Partitional calorimetric data and their physiologic correlates have been presented to show the thermal exchanges and responses of three acclimatized men, nude and clothed, while standing and walking in a series of seven hot environments (90° to 120° F.). In each of the environments the effects of varying the wind velocity between 30 and 600 feet per minute were determined. Convective and radiant heat gain and the compensatory evaporative heat loss showed a progressive increase with increasing air movement.

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FURTHER STUDIES OF THE EFFECTS OF INSULIN ON THE METABOLISM OF VITAMIN C¹

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In studying the metabolism of ascorbic acid in normal and depancreatized dogs it was found that although the animals in both groups synthesized vitamin C and their tissues were saturated with the vitamin, as measured by test doses, the urinary excretion of ascorbic acid for a 24-hour period was significantly and consistently lower in depancreatized dogs receiving insulin than in normal dogs. The major factor responsible for this difference in vitamin C excretion between the normal and the diabetic dogs proved to be the daily injections of insulin necessary to control the glycosuria in the depancreatized animal. When the insulin injections were stopped in the diabetic dogs there was a prompt increase in urinary excretion of the vitamin, the total vitamin excretion closely approximating the daily vitamin excretion prior to depancreatization. Furthermore, insulin injections caused a prompt fall in plasma concentration and urinary excretion of ascorbic acid in both normal and diabetic dogs (1). The present re-

port is concerned with an investigation of the mechanisms involved in this reaction.

I. THE RELATIONSHIP OF THE CHANGE IN PLASMA CONCENTRATION OF ASCORBIC ACID TO THE URINARY EXCRETION OF VITAMIN C

A constant intravenous infusion containing creatinine was given to a diabetic dog. Urine was collected by means of an indwelling catheter and preserved with sulfuric acid and hydroxyquinoline (2, 3). Blood samples were taken at frequent intervals. Regular insulin was injected once the blood level of creatinine and vitamin C were constant. Vitamin C was determined in the urine by the method of Evelyn *et al.* (4). Plasma was analyzed for ascorbic acid according to the method of Mindlin and Butler (5).

The results are given in Table I and show that the reabsorption of the vitamin is unaffected in the presence of the falling plasma concentration produced by the administration of insulin. The results indicate that the fall in urinary excretion of vitamin C following the injection of insulin is secondary to a fall in the plasma concentration of the vitamin and not due to a primary effect on the renal mechanism.

TABLE I
Effect of insulin on the renal mechanism for the reabsorption of ascorbic acid in a diabetic dog

Dog No.	Status	Time	Vit. C urine	Vit. C plasma	Glucose	Clearances			Vitamin C reabsorption	
						Vit. C	Creat.	Ratio vit. C creat.		
211	Diabetic	min.	mgm. %	mgm. %	mgm. %				mgm./min.	mgm./100 cc. filtrate
		0								
		11	2.65	0.66	411	23.48	56.8	0.41	0.22	0.47
		22	2.47	0.69	410	23.04	58.6	0.39	0.25	0.42
		34	2.60	0.72	408	20.55	58.8	0.33	0.28	0.47
Insulin 20 units I.V.										
		60	1.20	0.59	373	8.36	55.3	0.15	0.28	0.50
		78	0.36	0.49	328	2.45	57.9	0.04	0.27	0.47
		92	0.23	0.45	262	1.69	51.6	0.03	0.22	0.43

¹This research was aided by a grant from the Josiah Macy, Jr., Foundation.

II. EFFECT OF DIFFERENT TYPES OF INSULIN AND THE RELATIONSHIP BETWEEN THE EFFECT OF INSULIN AND THE ROUTE OF ADMINISTRATION

In the majority of the experiments regular insulin was used. To rule out the question of whether impurities might be the cause of the response to insulin, crystalline insulin was given in several experiments. This produced a rapid fall in the plasma ascorbic acid concentration similar to that caused by regular insulin.

Experiments in which the insulin was given intravenously, intramuscularly and subcutaneously showed that following the intravenous administration the effects on the plasma level of the vitamin occurred within 15 to 20 minutes, with a maximum effect at about 30 minutes; when injected intramuscularly the effect began in $\frac{1}{2}$ to 1 hour with a maximum effect reached in from 2 to 3 hours; and when the insulin was given subcutaneously the effect began after 1 to 2 hours, the maximum effect was reached after 4 to 5 hours, and the duration of the effect was about 6 to 7 hours.

With the insulin doses of 10 to 40 units used in the experiments, there was no correlation between the amounts of insulin and the changes in vitamin C levels. This is not surprising as it has been observed that the duration of action of insulin on carbohydrate metabolism is not proportional to the size of the dose injected but is a function of the logarithm of the dose (6).

III. THE MECHANISMS BY WHICH INSULIN MAY CAUSE THE CHANGES OBSERVED IN THE PLASMA LEVEL AND URINARY EXCRETION OF VITAMIN C

The mechanism by which insulin caused a depression in the plasma level and urinary excretion of vitamin C remained to be investigated. The most likely explanations seemed to be either (a) that insulin might have a direct action on ascorbic acid catalyzing its rate of oxidation or destruction; (b) that in the dog insulin might block the synthesis of ascorbic acid; (c) that the action of insulin on carbohydrate metabolism or other intermediary metabolites might involve ascorbic acid entering into and accelerating the oxidation or utilization of these metabolites in the body and in that way increasing the utilization of vitamin C; or (d) that insulin might cause a redistribution of ascorbic acid within the body. A

series of experiments were done to study these points.

(a) *Action of insulin on ascorbic acid in vitro:*

4 cc. of dog plasma were diluted with an equal amount of distilled water containing 0.8 unit of insulin. This was allowed to stand at room temperature for $\frac{1}{2}$ hour before precipitating with 8 cc. of 10% metaphosphoric acid. Another 4 cc. of plasma diluted with distilled water but with no insulin added was treated in a similar manner. Determinations of the vitamin C content were done on both solutions. The solution containing the insulin had 0.49 mgm. % of vitamin C and the control solution contained 0.52 mgm. % of the vitamin. Apparently insulin had no effect. In another experiment 30 cc. of whole blood was withdrawn from a normal dog and divided into three 10 cc. specimens. To the first specimen, 1 cc. of N/saline was added and the whole blood ascorbic acid concentration determined by the method of Butler and Cushman (7). To the second specimen, 1 cc. of N/saline was added and the whole specimen was then incubated at 37° for 1 hour before determination of the blood ascorbic acid. To the third specimen, 1 cc. of N/saline containing 0.6 unit of insulin was added and the specimen was incubated for 1 hour at 37° C. The concentrations of ascorbic acid of each of the three specimens were 0.6 mgm. %.

The results show that the insulin had no direct effect *in vitro* on the ascorbic acid in whole blood or plasma.

(b) *Action of insulin in blocking the synthesis of ascorbic acid:*

Since the fall in the plasma concentration and urinary excretion of vitamin C following the injection of insulin was always rapid, especially when the insulin was given intravenously, it seemed unlikely that such a rapid effect would be produced simply by blocking the synthesis of the vitamin. Further evidence against this hypothesis of insulin action on vitamin C metabolism was obtained by experiments done on man, who is incapable of synthesizing the vitamin, and on dogs. Several male diabetic patients served as the human subjects. The normal fasting variations in the plasma concentration of vitamin C were determined in each diabetic patient at intervals of $\frac{1}{2}$ hour for a 3-hour period. After an interval of several days the effect of injecting insulin on the plasma and urine levels of the vitamin was determined. A total of 10 experiments were done on the patients. Without insulin the plasma concentration of vitamin C did not vary by more than 0.10 mgm. % over the 3-hour period.

Following the injection of insulin there was a prompt and significant fall in the plasma concentration of the vitamin. The urinary excretion of the vitamin paralleled the changes in the plasma

TABLE II

The effect of oral glucose and of insulin on the plasma vitamin C levels of two diabetic patients

Time	Plasma vitamin C	Blood sugar	Time	Plasma vitamin C	Blood sugar
min.	mgm. %	mgm. %	min.	mgm. %	mgm. %
0	1.39	552	0	0.82	
33	1.37	544	33	0.90	177
[Glucose—100 gm.—orally			Glucose—100 gm.—orally		
25	1.41	624	18	0.86	308
43	1.37	648	38	0.91	372
62	1.35	656	56	0.84	348
78	1.32	704	72	0.83	320
97	1.38	672	91	0.83	300
118	1.35	600	108	0.82	292
Subject	Sz.		Subject	Sp.	
Time	Plasma vitamin C	Blood glucose	Time	Plasma vitamin C	Blood glucose
min.	mgm. %	mgm. %	min.	mgm. %	mgm. %
0	1.24		0	1.36	450
29	1.19	394	37	1.36	424
65	1.22	378			
Insulin 12 units I.V.			Insulin 20 units I.M.		
31	1.04	298	28	1.36	378
62	1.10	240	60	1.12	296
92	1.18	220	89	1.16	275
			Insulin 6 units I.V.		
			22	1.18	186
			52	1.04	105

levels. The average fall of plasma ascorbic acid in 10 experiments was 0.22 ± 0.07 mgm. %. In Table II are given the effects of insulin on the plasma vitamin C levels of two of the diabetic patients studied. It will be also noted that glucose alone had no effect on plasma vitamin C levels.

In another experiment a normal female dog, weighing $9\frac{1}{4}$ kg., was studied before and while receiving daily injections of insulin. The study was divided into four periods. In the first period of 7 days, the dog received no insulin; in the second period of 6 days, the animal was given one dose of 5 units of insulin subcutaneously daily; in the third period of 7 days, 10 units daily; and in the fourth period of 6 days, 15 units of insulin. Blood samples were drawn every other day prior to the injection of insulin. Daily 24-hour urinary excretions of ascorbic acid were determined.

The results of this experiment are given in Table III. The 24-hour excretion and fasting plasma concentrations of vitamin C in this normal dog did not show a decrease in any of the periods, although in acute experiments this same animal responded to injections of insulin with a fall in plasma concentration. If the 24-hour excretion of ascorbic acid is any indication of the synthesis of the vitamin in the dog, then over the 24-hour period insulin did not seem to affect the synthesis of the vitamin.

TABLE III

Effect of insulin on the 24-hour urinary excretion of vitamin C in the normal dog

No. of days	Units of insulin daily	Average 24 hr. excretion vitamin C	Fasting plasma vitamin C average
		mgm.	mgm. %
7	none	67	0.44
6	5	70	0.44
7	10	72	0.47
6	15	71	0.48

(c) *Possible action of insulin in increasing the utilization of vitamin C:*

We have presented some seemingly paradoxical data: *i.e.*, injected insulin in the normal dog causes a rapid but transient fall in plasma concentration and urinary excretion of ascorbic acid; yet the 24-hour excretion of ascorbic acid in the same dog seems unaffected by the administration of insulin. This is explained by the finding that after the acute fall of urinary vitamin C following insulin injections there is not only a return to normal but an increased excretion of the vitamin over the expected normal excretion. In another series of experiments the immediate and after effects of insulin on ascorbic acid metabolism were studied. The plasma concentration of ascorbic acid and the urinary excretion were followed hourly for periods of 10 hours. Table IV shows typical experiments. Similar findings were noted in both normal and diabetic dogs and show that, although the immediate effect of insulin is to cause a fall in plasma concentration and urinary excretion of the vitamin, once the immediate effect of the insulin has worn off there is an increased excretion of ascorbic acid for several hours and then a final return again to normal. The changes in the urinary excretion were paralleled by changes in the plasma

TABLE IV

Experiments showing the immediate and after effects of insulin on the vitamin C levels at hourly intervals
Dog No. 227 normal

Hours	0-1		1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Plasma vitamin C mgm. %	0.42	Insulin 10 units subcutaneously	0.31	0.22	0.28	0.35	0.45	0.49	0.45		0.44
Excretion vitamin C mgm./hour	4.1		2.2	1.3	2.0	2.8	5.9	13.4	9.6	6.4	4.5
Plasma glucose mgm. %	84		62	28	33		36	61	84	95	97

Dog No. 241 diabetic

Hours	0-1		1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Plasma vitamin C mgm. %	0.57	Insulin 20 units subcutaneously	0.45	0.35	0.35	0.44	0.60	0.71	0.74	0.66	0.60
Plasma glucose mgm. %	256		170	74	58		48		74		108

levels of the vitamin. The latter were not as striking as the urinary changes but occurred consistently in all the animals studied. Although this explains the findings in the normal dogs, it does not explain why the diabetic dog over 24-hour periods excretes less vitamin C than does the normal dog.

In order to test the hypothesis of increased utilization after injections of insulin, a series of experiments were done on rats, humans and guinea pigs. Five normal albino rats weighing 250 to 300 gms. were placed in individual metabolism cages suspended on large funnels containing a fine mesh filter. They were fed a Purina chow diet (which is devoid of ascorbic acid) and allowed water *ad lib*. 24-hour urines were collected into dark bottles containing sulfuric acid and hydroxyquinoline. The urines were analyzed for their vitamin C content. Each animal acted as its own control. The daily excretion of ascorbic acid was observed on diet alone for 7 days; then for the next 8 days insulin was injected once daily starting with 0.2 unit subcutaneously and increasing the dose by 0.1 unit each day, so that on the 8th day each animal received 0.9 unit of insulin. The animals were then observed for 7 days longer under conditions similar to the control period.

The results of this experiment are given in Table V. They show that insulin in the doses used had no effect on the 24-hour urinary excretion of ascorbic acid. It is concluded that in the normal rat, insulin does not increase the utilization of ascorbic acid.

Experiments were also done on human subjects. As previously stated man is incapable of synthesizing ascorbic acid and as the fecal excre-

tion of the vitamin is fairly constant and minimal (8) the 24-hour urinary excretion of the vitamin affords a measure of the difference between the daily intake and the metabolism or storage of vitamin C (9). The question of storage can be controlled by saturating an individual with the vitamin and maintaining him on a large dose daily, under which conditions the difference between

TABLE V

Effect of insulin on the daily urinary excretion of vitamin C in albino rats on the Purina chow diet

Rat No.	Age	Wt.	No. of days	Average 24-hr. urinary excretion vitamin C	Insulin* units
	days	gm.		mgm.	
1	112	277	7	1.2	none
		288	8	1.3	0.2-0.9
		299	8	1.4	none
2	112	273	7	4.1	none
		273	8	2.1	0.2-0.9
		267	7	2.4	none
3	112	286	7	5.0	none
		292	8	4.6	0.2-0.9
		293	7	5.6	none
4	112	260	7	2.3	none
		270	8	2.2	0.2-0.9
		283	7	2.5	none
5	112	262	7	3.4	none
		285	8	2.0	0.2-0.9
		285	7	2.2	none

* Rats were started on 0.2 unit of insulin daily and this was increased 0.1 unit daily.

TABLE VI

Effect of insulin on the 24-hour urinary excretion of vitamin C in a normal adult male on 200 mgm. of vitamin C daily

No. of days	Units of insulin daily	Average 24-hr. urinary excretion of vitamin C		Average fasting plasma vit. C
		mgm.	s.d.	
9	none	70	± 11	1.21
14	10 u.—1 day 15 u.—1 day 20 u.—1 day 30 u.—3 days 35 u.—2 days 40 u.—2 days 45 u.—4 days	84	± 18	1.12
4	none	83		1.18

the daily intake and excretion of vitamin C is a measure of the utilization.

A normal adult male, who in previous experiments had shown a fall in the plasma level of vitamin C following injections of insulin, was maintained on a diet free of vitamin C. At the start of the experiment he was given large doses of crystalline vitamin C orally (7,100 mgm. in an 8-day period) in order to assure saturation of his tissues, and then was maintained on a daily dose of 200 mgm. Twenty-four hour urine samples were collected for determination of vitamin C and the plasma

levels were done three times weekly. After a suitable control period insulin was injected subcutaneously daily beginning with 10 units and increasing to 45 units daily. The observations were continued for a short period following the withdrawal of insulin.

The results of this experiment are given in Table VI. The injections of insulin did not significantly affect the 24-hour urinary excretion of ascorbic acid, which would indicate that insulin does not increase the rate of utilization of ascorbic acid in man. This fact was further demonstrated in an experiment on guinea pigs, the results of which are given in Table VII. The onset of the signs of scurvy and the severity of the lesions at autopsy were not affected when insulin was given daily to the animals on the vitamin C-free diet. The evidence cited in experiments on dogs, rats, humans and guinea pigs does not suggest that insulin produces the acute effects on vitamin C levels through an increased utilization of the vitamin resulting in its loss from the body.

(d) *Does insulin cause a redistribution of vitamin C in the body tissues?*

None of the theories so far discussed adequately explains the acute effects of insulin on the plasma

TABLE VII

*Effect of the daily injection of insulin in guinea pigs on a scorbutic diet**

Pig No.	Units of insulin daily	Weight gms.		Day of onset of severe scurvy	Day of death	Grading of autopsy findings†							
		Onset	Final			Bony system changes				Hemorrhages			
						Long bones	Teeth	Ribs	Joints	Ribs	Intest.	Joints	Muscles
1	none	310	220	14	30†	4+	3+	2+	3+	1+	2+	2+	4+
2	none	290	303	14	23	1+	1+	2+	2+	2+	4+	3+	3+
3	none	268	330	13	28	2+	2+	2+	2+	3+	3+	3+	4+
8	none	330	253	22	30†	4+	1+	4+	3+	3+	4+	4+	4+
4†	0.6	281	194	16	30†	2+	4+	2+	3+	2+	1+	3+	3+
5†	0.6	288	250	15	26	2+	4+	2+	3+	1+	1+	3+	4+
6†	0.6	350	331	15	30†	2+	4+	4+	2+	2+	1+	4+	4+
7†	0.6	282	209	15	26	3+	4+	2+	1+	2+	4+	2+	2+
9†	0.6	326	325	18	30†	4+	1+	2+	3+	1+	1+	3+	4+

* The diet consisted of rolled oats 39%, bran 20%, skimmed milk powder (heated to 110° C) 30%, butter fat 10% and NaCl 1%. Each pig received 1 drop of Cod Liver Oil daily.

† The pigs on insulin were started on 0.2 units daily. This was increased 0.2 units daily to a dose that caused symptoms of shock and the pig was then carried on $\frac{2}{3}$ of that amount.

‡ These pigs were sacrificed and the vitamin C content of the plasma was determined. In every instance it was zero.

§ The findings at autopsy were graded as follows: 1+ (mild changes), 2+ (moderate), 3+ (severe), 4+ (very severe). For the bony system, the findings of looseness of the teeth, fragility of bones, spontaneous fractures of the long bones, enlargement and hemorrhages of the joints and ribs, and separation of the epiphyses were graded. For hemorrhages, the number and extent of hemorrhages were graded.

level and urinary excretion of vitamin C. It seemed to us that a satisfactory explanation of the facts might be that insulin causes a redistribution of ascorbic acid in the body: *i.e.*, that the vitamin passes from the plasma into the tissues or tissue fluids and that, as the effect of insulin wears off, the vitamin again returns to the plasma. The proof of such an hypothesis would depend on demonstrating that coincident with the fall in plasma concentration of vitamin C there occurred a rise in concentration in the tissues or in some specific tissue and that the changes in concentration would quantitatively balance one another.

Quantitatively accurate determinations of the vitamin C content of muscles and organs under the conditions necessary in these experiments were not practical. However, the methods for determining the vitamin C content of whole blood, red blood cells and the white blood cell-platelet layer of the blood as well as the plasma were satisfactory and therefore the determination of these components of the blood were done before and after the administration of insulin. Control blood samples were taken and, following the injection of insulin, samples were collected at suitable intervals depending on the mode of administration of the insulin. Eleven experiments were done and the results are shown graphically in Figure 1 and the details are given in Table VIII. The methods

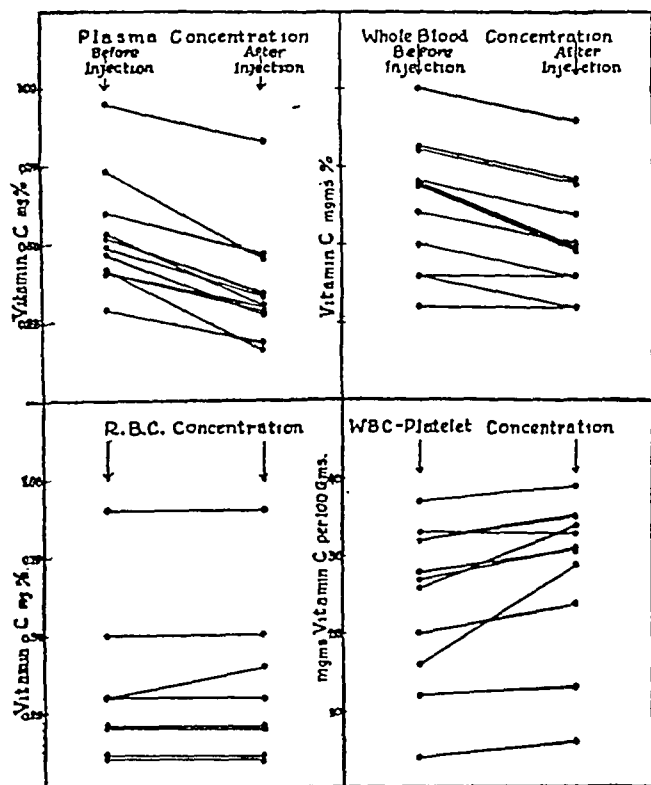


FIG. 1. CONCENTRATION OF VITAMIN C IN BLOOD CONSTITUENTS BEFORE AND AFTER INSULIN

of determining the vitamin C concentration in the various blood fractions were those described by Butler and Cushman (7). In 6 experiments hematocrit determinations were made and the calculated and observed values for the whole blood ascorbic acid were found to agree closely.

In all of the 11 experiments there was a fall in plasma concentration of ascorbic acid after the injection of insulin. In 10 of the 11 experiments the fall can be considered significant. In one experiment the fall was within the extremes of normal variation. Of 8 experiments in which the R.B.C. concentration of ascorbic acid was determined (7 directly and one by calculation), there was no change in R.B.C. concentration of the vitamin in 7 experiments. In one experiment there was a slight rise in concentration of vitamin C following injection of insulin. In 9 of 10 experiments there was a rise in the concentration of ascorbic acid of the buffy layer (W.B.C.-platelet) after insulin; 4 of the increases were definitely significant (over 3 mgm. %), while 5 were not greater than the range of error of the method. The average determined increase after insulin in the buffy layer concentration for the 10 experiments was 4 mgm./100 gms. leukocytes. In 6 experiments calculation of the W.B.C.-platelet layer concentration of ascorbic acid from the determined hematocrit, whole blood, plasma and red blood cell concentrations showed an average rise of 5 mgm./100 gms. leukocytes following insulin administration. We think the changes are significant. There was a slight fall in whole blood concentration of vitamin C in 9 experiments. In the other two no change was noted. The results suggest that after the injection of insulin there is a fall in the plasma and the whole blood concentration of ascorbic acid; no change in the red blood cell ascorbic acid content; and a rise in the vitamin C concentration of the W.B.C.-platelet layer.

DISCUSSION

The results of the series of experiments reported here and previously (1) show that following the injection of insulin into a normal or diabetic dog or a human normal or diabetic subject, there results a fall in plasma concentration and an attendant drop in the urinary excretion of vitamin C; that this effect wears off after a variable period of time and is followed by a rise in plasma concen-

TABLE VIII

The effect of insulin on the concentration of vitamin C in the various elements of the blood

Experiment No.	Animal	Units of insulin given	Time after insulin	Vitamin C plasma	Vitamin C red blood cells	Vitamin C W.B.C.—platelet layer	Vitamin C whole blood	Blood sugar
				mgm. %	mgm. %	mgm./100 gm.	mgm. %	mgm. %
1	Dog 232	20 I.V. 15 I.V.	Fasting 1 hour	0.41 0.30	0.1 0.1	27 31	0.6 0.5	
2	Human diabetic patient	35 I.V.	Fasting 45 min.	0.95 0.83	0.9 0.9	32 35	1.0 0.9	303 123
3	Dog 234	20 Subcut.	Fasting 2 hours	0.49 0.33		26 34	0.8 0.7	
4	Dog 236	20 Subcut.	Fasting 2 hours	0.42 0.17†		12 13	0.4 0.3	
5	Dog 234	20 Subcut.	Fasting 2 hours	0.60 0.47			0.7 0.5	Dog in Shock
6	Dog 234	20 I.V.† 20 I.M.†	Fasting 45 min.	0.53 0.31	0.2* 0.2*	33 33	0.5 0.4	98 40
7	Dog 234	20 I.V. 30 I.M.	Fasting 1 hour	0.52 0.34	0.3 0.4	37 39	0.7 0.6	
8	Dog 234	25 I.V. 25 I.M.	Fasting 1 hour	0.73 0.46	0.3 0.3	16 29	0.8 0.7	
9	Dog 227§	15 I.V. 20 I.M.	Fasting 50 min.	0.29 0.19	0.1 0.1	4 6	0.3 0.3	
10	Dog 231	20 I.V. 20 I.M.	Fasting 50 min.	0.41 0.29	0.2 0.2	20 24	0.4 0.4	
11	Dog 234	20 I.V. 10 Sub.	Fasting 45 min.	0.47 0.28	0.5 0.5	28 31	0.7 0.5	89 33

* By calculation from hematocrit.

† Crystalline insulin.

‡ Slight hemolysis present.

§ Dog had lost considerable weight and was severely diabetic.

tration and an increased excretion of ascorbic acid, and finally by a return to the normal levels. The results indicate that there is no loss of ascorbic acid from the body and suggest that vitamin C may be redistributed within the tissues coincident with some metabolic action of insulin. One would be tempted to relate this redistribution to changes in carbohydrate metabolism; but no evidence to this effect was found in the experiments done. Associated with the effect of insulin was a transfer of ascorbic acid from the plasma to the W.B.C.-platelet layer of the blood. The ability of vitamin C to shift rapidly from the plasma to the W.B.C. layer has been demonstrated both *in vitro* by Heinemann (10) and *in vivo* by ourselves in experiments on leukemic patients (11). The *in vitro* transfer of vitamin C from the W.B.C. layer to plasma is reported to be a slow process (12).

There is much indirect evidence that the W.B.C.-platelet layer concentration of ascorbic acid is an index of tissue concentration (13, 14). Since we noted a small increase in the buffy layer concentration after insulin injections, and as this by calculation only accounted for a small per cent of the temporary plasma loss of ascorbic acid and for none of the whole blood loss, it is suggested that vitamin C passes from the plasma and extracellular compartment not only into the W.B.C.-platelet layer but into the tissues as well and that it is eventually returned to the plasma. Our experiments indicate that the action does not seem to involve an actual utilization of ascorbic acid, but rather suggest that it may have some catalytic function. Haid (15) has noted a noticeable decrease in ascorbic acid content of the blood after large doses of insulin. He suggests that vitamin C wanders from the blood stream into the tissues,

particularly the liver, and also suggests that there exists a close relationship between vitamin C and carbohydrate metabolism. Göbell and Krause (16) found that the administration of insulin and also adrenalin and nicotinamide lowered the content of reduced and dehydroascorbic acid in the blood. They suggested, as we have, that the increased metabolism in tissues temporarily deflected ascorbic acid from blood to tissues and that, after the stimulus to metabolism was over, vitamin C returned to the blood having fulfilled its role as a catalyst. If ascorbic acid does have some catalytic function in intermediary carbohydrate metabolism then one might expect to find a decreased carbohydrate tolerance in scorbutic subjects and improvement in tolerance following vitamin C therapy. This has been reported in the scorbutic guinea pig by Sigal and King (17). The literature on the relationship of ascorbic acid to carbohydrate metabolism has been confusing due to the fact that most of the observations have been on animals capable of synthesizing ascorbic acid (dogs, rabbits and rats) whose tissues are probably saturated with the vitamin, or on humans in whom the state of vitamin C nutrition has not been taken into account. (Studies of carbohydrate metabolism in human scurvy and severe ascorbic acid deficiency states, before and after vitamin C therapy, would be interesting.)

Insulin has been repeatedly demonstrated to cause a fall in plasma inorganic phosphate (18), potassium (19) and uric acid (20). It may well be that factors governing the above changes may also play a part in the changes in ascorbic acid noted.

SUMMARY

The mechanism by which insulin causes a prompt fall in plasma and urinary vitamin C levels was investigated. It was found that insulin caused these changes regardless of whether it was injected subcutaneously, intravenously or intramuscularly. There were, however, significant differences in both the onset and duration of the effects of insulin on both the plasma levels and excretion of vitamin C depending on the route of administration of the insulin. The insulin effects in each instance were transient and were followed by a period in which the plasma level and the uri-

nary excretion were elevated. This was followed by a return of the vitamin C levels to normal.

In all species studied—man, dog and rats—these changes in vitamin C occurred in the plasma and urine following injections of insulin.

Studies in dogs, rats, guinea pigs, and humans, and determinations of the vitamin C content of blood constituents directed toward establishing the mechanism of the action of insulin on vitamin C metabolism showed: (a) that insulin had no effect *in vitro*; and (b) that insulin did not affect the synthesis of ascorbic acid. It appears that insulin causes a fall in plasma and whole blood ascorbic acid content; exerts no effect on R.B.C. ascorbic acid content; and is associated with a rise in the W.B.C.-platelet layer concentration of vitamin C.

It is suggested that insulin in its action or actions causes a transient transfer of ascorbic acid from the plasma and extracellular fluid into the tissues, possibly for some catalytic function of ascorbic acid.

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THE ABSORPTION OF ORALLY ADMINISTERED EMULSIFIED LIPID IN NORMAL CHILDREN AND IN CHILDREN WITH STEATORRHEA^{1,2}

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INTRODUCTION

Vitamin A is a lipid whose identification in the peripheral blood is relatively simple. We have used this substance to study absorption of lipids from the intestine. The observation was made that oily preparations of vitamin A appeared to be less easily absorbed than a preparation intended to be mixed with ordinary infants' formulae (1). It occurred to us that this might be due to the vitamin A being in an emulsified state when mixed with the formulae. The experiments herein reported were conducted with two purposes in mind: (1) to determine whether an orally administered emulsified lipid is absorbed better than an unemulsified lipid in individuals with impaired absorption of fat, and (2) to investigate the validity of the Frazer "Partition Hypothesis" for lipid absorption (2).

Frazer's "Partition Hypothesis" states that lipids may be absorbed from the gastrointestinal tract without lipolysis. His theoretical considerations are substantiated by feeding marked fats, by chylomicron counts on the peripheral and portal blood, and by direct observation of the intestine and lacteals of animals during acute experiments. Sinclair (3) has offered evidence in support of the Vezer theory of fat absorption (lipolysis and phosphorylation). Fats foreign to the normal animal organism have been identified following ingestion as part of the phospholipids of the intestinal mucosa. It would seem that both mecha-

nisms of absorption of fat may occur. The quantitative contribution of each mechanism has not been ascertained.

PLAN OF STUDY

Vitamin A absorption curves were obtained in three groups of children using a procedure previously reported (4). The plasma vitamin A levels are reported in arbitrary galvanometer units (L—620 units per 100 cc. plasma). In all the experiments the test dose of vitamin A preparation was given after a 12-hour fast and followed by 300 cc. of whole cow's milk or a normal breakfast.

The first group was composed of 14 normal children convalescent from acute infections. The second group of absorption curves was obtained from 14 children with fibrosis of the pancreas in whom it had been demonstrated that tryptic activity was deficient in the duodenal secretions. Lipase is also deficient in these children because of the complete destruction of the exocrine portion of the pancreas. The third group of absorption curves was obtained from 6 children with steatorrhea due to the "celiac" type of disorder.

The materials used in the experiments are listed in Table I. Unemulsified vitamin A (I), largely in ester form, derived from fish liver oils was administered to these three groups of children. An emulsion composed of 15 per cent vitamin A acetate, 15 per cent Tween 20⁵ and 70 per cent water (II) was prepared in which the oil droplet size varied from 0.5 to 10.0 microns; this too was administered to these three groups of patients. Also given to 3 normal children was a standard test dose of unemulsified vitamin A esters accompanied by 10 grams of pancreatin (III). Two normal children received 4 cc. of Tween 20 alone (IV).

To a group of 5 children with fibrosis of the pancreas, a test dose of unemulsified vitamin A esters were administered accompanied by 5 to 20 grams of commercial

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⁵ The Tweens (Atlas Powder Co.) are a class of non-ionic surface-active agents. The hydrophylic portion of the molecule is composed of free hydroxy groups and an oxyethylene chain. The lypophilic portion in the case of Tween 20 is lauric acid. The Tweens are excellent emulsifying agents and as far as is known these compounds are not toxic when fed to animals or humans.

TABLE I

Materials used in experiments

I. Unemulsified vitamin A esters (Mead Johnson and Company: Oleumpercomorphum)	60,000 I.U. Vitamin A/gram 8,500 I.U. Vitamin D/gram
II. Vitamin A acetate emulsion (Prepared by Abbott Research Laboratories, Inc.)	15% Vitamin A acetate 15% Tween 20 70% Water
III. Unemulsified vitamin A esters plus pancreatin (Parke Davis and Co.: Enteric Granules)	3 times U.S.P. trypsin activity. Lipase activity not specified but similar preparations have been found to be very low in lipolytic activity (12)
IV. Polyoxethylene sorbitan monolaurate (Atlas Powder Company: Tween 20)	
V. Emulsified vitamin A esters	33% Oleumpercomorphum 24% Tween 20 38% Water 5% Propylene glycol
VI. Propylene glycol (Glogau and Company, Chicago)	
VII. Crystalline vitamin A alcohol (Distillation Products, Inc., Rochester, N. Y.)	3,000 I.U. Vitamin A/milligram

pancreatin (III) and absorption curves obtained. Finely emulsified vitamin A esters (V) were administered to 3 children with fibrosis of the pancreas. Preparation V contained propylene glycol,⁶ and because some alcohols alone when administered by mouth are capable of producing a marked elevation in the plasma vitamin A (5), it was thought necessary to administer propylene glycol (VI) alone to exclude this phenomenon. This was performed on 5 normal controls.

⁶ Propylene glycol was used to enhance emulsification. Vitamin A esters are partially soluble in it, and it in turn is completely miscible with water.

The size of the visible particles in all these emulsions was determined microscopically by dark field and light field examinations of the oily films with an ocular micrometer disk.

RESULTS

These are summarized in Table II.

Normal children:

Unemulsified vitamin A in the form of oleumpercomorphum (I), when given to the normal

TABLE II

*Summary of vitamin A absorption experiments on normal patients, patients with fibrosis of the pancreas, and patients with steatorrhea**

Experimental material	Average particle size in microns	Normal patients			Patients with fibrosis of the pancreas			Patients with steatorrhea		
		Aver. max. rise of plasma vit. A	Range of plasma vit. A rise	No. of patients in experiment	Aver. max. rise of plasma vit. A	Range of plasma vit. A rise	No. of patients in experiment	Aver. max. rise of plasma vit. A	Range of plasma vit. A rise	No. of patients in experiment
I. Unemulsified vitamin A esters	0.5-10	(119)†	(52-283)†	(47)†						
II. Vitamin A acetate emulsion		258	89-557	8	7	0-15	7	40	6-88	6
III. Unemulsified vitamin A esters plus pancreatin		232	47-305	9	119	16-292	11	49	14-113	3
IV. Polyoxethylene sorbitan monolaurate	1	77	5-134	3	58	36-94	5			
V. Emulsified vitamin A esters		1	0-1	2						
VI. Propylene glycol					49	36-64	2			
VII. Crystalline vitamin A alcohol		0	0-1	5	104	94-114	2			

* All values for vitamin A are expressed in L-620 units per 100 cc. of plasma.

† May (13).

group of children, produced rises in the plasma vitamin A which were quite comparable to those which had been previously reported (6). Vitamin A acetate in Tween 20 (II), given to the same group of normal children, produced a similar though greater elevation in plasma vitamin A. As shown by the data in Table II, Tween 20 administered alone to 3 normal children produced no change in the plasma vitamin A. The 3 normal children given vitamin A esters plus pancreatin (III) gave normal curves in two instances and a lowered curve in the third. The administration of propylene glycol (VI) alone to the normal group in amounts similar to those given with the emulsified vitamin A esters (V) produced no change in plasma vitamin A.

Fibrosis of the pancreas:

Children in whom the diagnosis of fibrosis of the pancreas had been made were given unemulsified vitamin A esters in the form of oleumpercomorphum (I), and the maximum rise of plasma vitamin A was, as may be seen in Table II, extremely low. When vitamin A was given to this group as vitamin A acetate in Tween 20 (II) and as emulsified vitamin A esters, a rise in plasma vitamin A occurred of about the extent observed in the normal controls. When unemulsified vitamin A esters were given with pancreatin (III) to patients with fibrosis of the pancreas, considerable increase in plasma vitamin A was noted, but the rises did not approach those obtained either in the normal group or in the same patients with the other emulsified forms of vitamin A.

The magnitude of response was not uniform in all the children with fibrosis of the pancreas. Two of the 11 patients given vitamin A acetate in Tween 20 showed little rise in plasma vitamin A. All the patients given pancreatin plus vitamin A showed consistent responses.

Steatorrhea:

Children with steatorrhea were given vitamin A acetate emulsion (II), and the results as seen in Table II were quite comparable to those obtained with children who had fibrosis of the pancreas with the exception that the magnitude of elevation was slightly less.

DISCUSSION

The absorption curves in normal children serve only to demonstrate that normal individuals absorbed the tested materials well, and that neither propylene glycol nor Tween 20 was *per se* capable of eliciting an elevation in plasma vitamin A. Of much greater interest were the results obtained in children with fibrosis of the pancreas. All preparations of vitamin A which resulted in improved absorption were found *in vitro* to be made up of droplets 0.5 to 10 microns in diameter and may be assumed to have presented this lipid to the intestine in a fine particulate state. According to Frazer's hypothesis, this would account for the improved absorption in the absence of lipase. Indeed these experiments may be considered to offer further evidence in support of Frazer's hypothesis. It would be difficult to explain these results by the classical lipolysis theory of fat absorption.

Vitamin A in its crystalline form is an alcohol. In fish liver oils it is largely esterified with high molecular weight fatty acids. As an ester its digestion has been thought to follow the route of other esters, such as neutral fats, and thus to be dependent upon lipolysis.⁷ Adlersberg and Sabotka (7) found that when vitamin A was administered with phosphatids to adults with steatorrhea, the amount of vitamin A absorbed per unit time was increased. Although they felt that the enhanced absorption was due to increased emulsification, they reasoned that one could not be sure that the increased emulsification did anything more than permit more rapid lipolysis. This explanation would seem to be invalid in children with fibrosis of the pancreas because they have no *pancreatic* lipase, although the possibility remains that traces of lipolytic activity may be present in the intestine derived from sources other than the pancreas. Possibly creation of an emulsion with particles of the appropriate size (0.5 micron) enables the gastrointestinal tract to absorb oily material independent of lipolytic digestion.

⁷ Crystalline vitamin A alcohol (VII) was administered to 2 children with fibrosis of the pancreas in a dose of 6,000 I.U. per lb. This was given mixed with applesauce. It is seen in Table II that normal absorption curves were obtained. Good absorption of crystalline vitamin A alcohol by children with fibrosis of the pancreas has also been reported by Clausen (9).

The explanation for the few instances of poor absorption in children with fibrosis of the pancreas or steatorrhea when given finely emulsified vitamin A remains obscure. It is possible that intestinal hypomotility could account for this. The poor absorption of dextrose in such patients has been found to be due *in part* to intestinal hypomotility (8).

The observation that the addition of pancreatin to unemulsified vitamin A produced considerable increase in its absorption by children lacking lipase also requires explanation. This observation has been previously made by Clausen (9). When Frazer (10) fed rats triglycerides marked with Sudan IV, he observed staining of the fat depots and a postprandial elevation of lipid in the systemic circulation. When this same material was fed with lipase, he noted neither systemic lipemia nor staining of the fat depots. Rather he observed a portal lipemia and staining of the liver sinusoids. When adult humans were fed triglycerides (11), he observed again a systemic lipemia, but administration of lipase with equal amounts of fat abolished this phenomenon. Frazer interpreted these observations to indicate that addition of lipase augmented hydrolysis and subsequent absorption by the portal route. It would seem that pancreatin, which is poor in lipase (12), when administered to the children with absent lipase, must act mainly as an emulsifying agent.

If the increased absorption of emulsified vitamin A indicates enhanced lipid absorption, the implications would seem significant. A principal difficulty in maintaining children with chronic intestinal insufficiency is to supply them with sufficient calories in a form easily available at a period when most needed. If one could administer fat in a form that could be absorbed independent of low lipase, intestinal hypomotility, or other causes of malabsorption, it is possible that considerable progress might be made in aiding the nutrition of these children. Finally, it is possible that the oral administration of emulsified lipids to children recuperating from diarrhea might be of benefit. Studies are now in progress on the effect of feeding emulsified fats to these types of children and determining the absorption of the fats by balance measurements.

SUMMARY

The oral administration of emulsified vitamin A to normal children produced an elevation of plasma vitamin A comparable to that obtained when unemulsified vitamin A was administered. When these same emulsified preparations were given to children with steatorrhea, whether due to absent lipase or to other causes, there resulted a considerable improvement of absorption of vitamin A to approximately normal. Administration of unemulsified vitamin A resulted in poor absorption. The addition of commercial pancreatin to unemulsified vitamin A produced considerable increase in absorption in children lacking pancreatic secretions, but of less extent than was gained by giving emulsified vitamin A. These results suggest an enhanced absorption of food lipids by children with steatorrhea when provided in emulsified form.

ACKNOWLEDGMENT

We are indebted to Dr. A. E. Osterberg of Abbott Research Laboratories, Inc., for the preparation and generous supply of emulsions of vitamin A acetate with Tween 20.

ADDENDUM

Since this report was presented, Kramer *et al.*⁸ have reported enhanced absorption of vitamin A esters, when mixed with wetting agents, in 5 patients with "celiac disease," and Lewis *et al.*⁹ in an excellent study of absorption of "aqueous preparations" of vitamin A reported enhanced absorption in one child with fibrosis of the pancreas.

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⁸ Kramer, B., Sobel, A. E., and Gottfried, S. P., Serum levels of vitamin A in children. *Am. J. Dis. Child.*, 1947, 73, 543.

⁹ Lewis, J. M., Bodansky, O., Birmingham, J., and Cohan, S. Q., Comparative absorption, excretion, and storage of oily and aqueous vitamin A. *J. Pediat.*, 1947, 31, 495.

TABLE I

Mean values for serum protein constituents in 30 normal human subjects contrasted with electrophoretic values for 20 samples of pooled plasma (6)

*Chemical values

	T.S.P.	"G.G."	Alb.	Glob.	A/G	A/T.S.P.	"G.G."/A	"G.G."/G	"G.G."/T.S.P.
Mean value	6.96	0.860	4.96	2.00	2.5	71.3	17.4	44.3	12.3
Standard deviation	0.37	0.144	0.26	0.38	0.6	4.4	3.0	7.2	1.6
Coefficient of variation in per cent	5.3	16.7	5.3	19.0	23.1	6.2	17.2	16.3	13.0

† Electrophoretic values (Armstrong <i>et al.</i> [6])									
Mean	(7.30)	0.803	4.03	2.79	1.4	55.2			11.0
Coefficient of variation						2			6

* Values expressed in grams per 100 ml. serum or as per cent.

† The values of Armstrong *et al.* (6) are expressed only in per cent, to indicate the proportion which each fraction comprised of the whole plasma protein. The value for total plasma protein of 7.30, used for the conversion of his percentages to grams per cent, was derived by adding a value of 0.304 gram for fibrinogen to our mean value for total serum protein.

tions of the "gamma globulin" during the course of a few specific illnesses.

Values for total serum protein, serum albumin and "gamma globulin" in normal subjects

Simultaneous determinations have been made in 30 normal subjects of the total serum protein (T.S.P.) serum albumin (A), total serum globulin (G) and of the globulin fraction precipitated with 33.3 per cent saturated ammonium sulfate which is referred to as G.G. 33.3. The serum albumin and total serum globulin were measured by the method of Howe (3) using 21.5 per cent sodium sulfate. The initial one-third of the albumin filtrate was discarded as recommended by Gutman and coworkers (4). The G.G. 33.3 was determined by our method, as already described (1). All proteins were measured colorimetrically by the dilute biuret method of Weichselbaum (2). This method was calibrated and checked frequently with determinations of protein nitrogen, using a semimicro-Kjeldahl technique (5). The factor 6.4 was used for conversion of nitrogen to total protein.

In the control subjects there were 18 males and 12 females ranging in age from 18 to 35 years. All blood specimens were obtained in the morning approximately 2 to 3 hours after breakfast. Venous stasis was minimized by applying a tourniquet only immediately before withdrawal of the blood. All patients were sitting when samples were withdrawn. The results are presented in Table I which also, for comparison, indicates the electrophoretic values obtained by Armstrong and coworkers (6) from 20 samples of pooled plasma.

It is difficult to compare our normal values for protein fractions with those obtained electrophoretically for normal plasma. The value for serum albumin is higher and the value for serum globulin

is lower with the Howe technique than the corresponding electrophoretic values (7 to 9). It has been demonstrated that the precipitation of serum with 21.5 per cent sodium sulfate does not remove all of the alpha and beta globulin (4, 7). Likewise, as we have indicated, our "gamma globulin" is not entirely pure and does not contain all the gamma globulin present in serum.

Weekly fluctuation in chemically determined protein fractions in normal control subjects

If one wishes to apply the chemical fractionation of proteins to clinical disorders, it is necessary to know the magnitude of fluctuation of these components from time to time in a given individual. In 5 healthy control subjects we have determined the total serum protein, serum albumin, total serum globulin and the G.G. 33.3 fraction many times at intervals of 1 to 3 weeks over a period ranging from 6 to 12 months. The observed fluctuations are indicated in Table II. It appears that the total globulin of serum exhibits the greatest range of variation while the total serum protein and the serum albumin seem to have the greatest stability.

Values for G.G. 33.3, serum protein and serum albumin in pathologic sera

In a variety of pathologic states, we have determined the protein fractions, using the methods previously described. In Table III only single determinations from a given individual were em-

TABLE II

Weekly fluctuations of chemically determined protein constituents in 5 normal subjects

Name	No. deter.	Total protein			"Gamma globulin"			Albumin			Globulin			Albumin/globulin		
		Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.
H. A.	21	6.66	0.31	4.7	0.903	0.089	9.9	4.62	0.24	5.2	2.07	0.38	18.4	2.3	0.6	26.1
V. J.	30	6.92	0.34	4.9	0.748	0.079	10.6	5.00	0.28	5.6	1.94	0.38	19.6	2.7	0.5	18.5
G. G.	23	6.81	0.30	4.4	0.813	0.095	11.7	4.66	0.26	5.6	2.14	0.32	15.0	2.2	0.4	18.2
V. D.	18	6.74	0.35	5.2	0.692	0.098	14.2	4.68	0.31	6.6	2.01	0.34	16.9	2.4	0.5	20.8
R. B.	13	6.68	0.34	5.1	0.680	0.068	10.0	4.82	0.19	3.9	1.87	0.34	18.2	2.7	0.7	25.9

Name	No. deter.	Albumin/T.S.P.			"G.G." / albumin			"G.G." / globulin			"G.G." / T.S.P.		
		Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.
H. A.	21	68.7	4.3	6.2	19.2	2.3	12.0	44.8	8.5	19.0	13.4	1.4	10.4
V. J.	30	72.2	4.5	6.3	15.2	1.5	9.9	40.9	9.7	23.7	10.8	1.2	11.1
G. G.	23	68.4	4.1	6.0	17.7	2.2	12.4	40.5	6.4	15.8	11.9	1.5	12.6
V. D.	18	70.1	4.0	5.7	15.4	2.0	13.0	36.4	5.2	14.3	10.3	1.2	11.7
R. B.	13	72.1	4.3	5.9	14.1	1.8	11.7	37.9	9.8	26.9	10.2	1.0	10.0

played to obtain the mean values and the total ranges for different disorders.

It will be noted that, in this group of disorders, there often was a reduction in serum albumin and a considerable increase in the G.G. 33.3 fraction as contrasted with normal values. In instances where the G.G. 33.3 fraction was increased greatly, there usually was an increase in total globulin.

However, the increase in total globulin was sometimes small and yet a substantial increase in the G.G. 33.3 fraction occurred, as in the case of the patients with beta hemolytic streptococcal pharyngitis.

Because of the variations in plasma volume which may occur during infections, it seems preferable when following the course of a specific dis-

TABLE III

Disease	Approximate duration	Number patients	T.P.	Alb.	Glob.	"G.G."	A/G	A/T.P.	"G.G." / A	"G.G." / G	"G.G." / T.P.
Normal controls		30	6.96	4.96	2.00	0.860	2.5	71.3	17.4	44.3	12.3
Streptococcal pharyngitis	7-14 days	13	7.27	4.73	2.55	1.280	2.1	65.3	27.2	55.0	17.8
Malaria inoculata	21-28 days	4	6.67	2.87	3.81	1.427	0.7	47.8	50.2	37.6	21.4
Chronic staph. osteomyelitis	months	1	7.10	3.81	3.29	1.850	1.2	53.7	48.7	56.5	26.2
Acute rheumatic fever	10-21 days	17	7.11	4.07	3.11	1.570	1.4	57.7	40.9	51.0	22.0
Gonococcal arthritis	10-30 days	3	7.00	3.88	3.12	1.212	1.6	55.2	31.5	38.8	17.3
Subacute bacterial endocarditis	months	2	A. 6.91 B. 6.39	3.40 4.02	3.51 2.37	1.365 1.526	1.0 1.7	49.1 63.0	40.0 38.0	39.0 64.4	19.8 23.9
Chronic rheumatoid arthritis	months-years	29	7.16	4.27	2.90	1.458	1.6	60.1	34.8	50.6	20.0
Erythema multiforme	14-21 days	3	7.40	4.07	3.37	1.617	1.2	58.7	39.4	47.7	21.5
Disseminated lupus erythematosus	unknown	3	5.68	2.45	3.56	1.901	0.7	48.3	62.3	59.2	28.5
Dermatomyositis	unknown	4	7.23	3.56	3.72	1.793	1.1	56.1	51.7	48.9	25.1
Cirrhosis of liver	unknown	9	6.57	3.92	3.38	1.591	1.0	48.6	51.2	47.8	21.9
Infectious hepatitis	6 weeks	1	8.17	3.86	4.31	1.625	0.9	47.2	42.0	37.6	20.0
Lymphogranuloma inguinale	unknown	3	8.52	4.23	4.28	2.547	1.1	50.3	61.5	58.5	29.1
Nephrotic syndrome	unknown	6	4.89	2.45	2.47	0.443	1.2	49.2	19.1	17.9	8.8

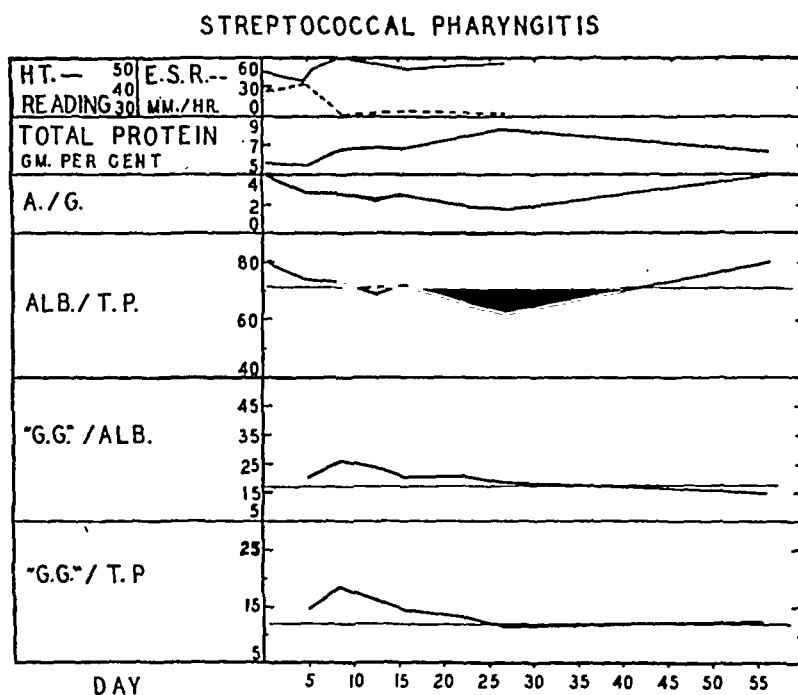


FIG. 1. NOTE THAT IN THIS UNCOMPLICATED CASE OF STREPTOCOCCAL PHARYNGITIS, PROTEIN ABNORMALITIES PERSISTED AFTER THE ERYTHROCYTE SEDIMENTATION RATE BECAME NORMAL

The mean values for the ratios of these protein constituents in normal subjects are indicated by fine horizontal lines.

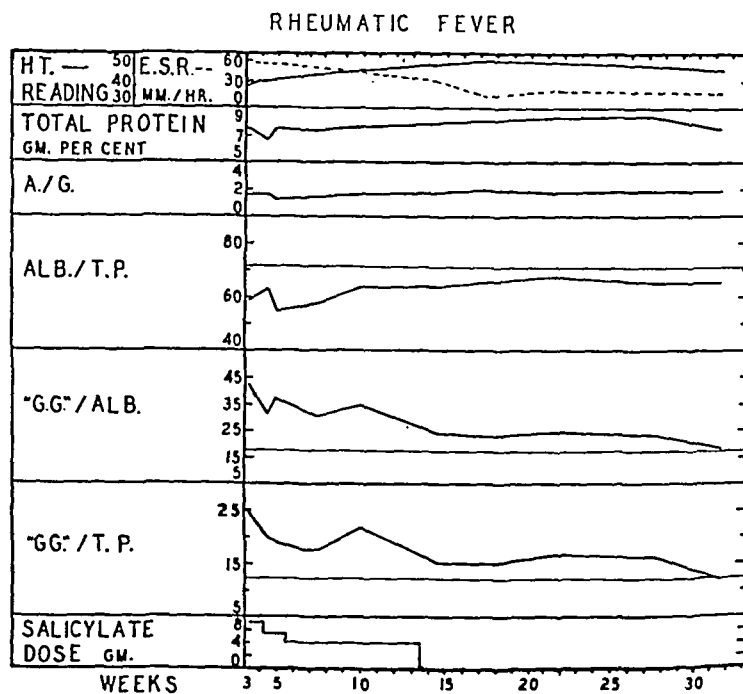


FIG. 2. IN THIS YOUNG ADULT WITH ACUTE RHEUMATIC FEVER, MODERATE ABNORMALITIES IN PROTEIN CONSTITUENTS PERSISTED FOR A PROLONGED PERIOD AFTER CLINICAL EVIDENCE OF ACTIVITY HAD SUBSIDED

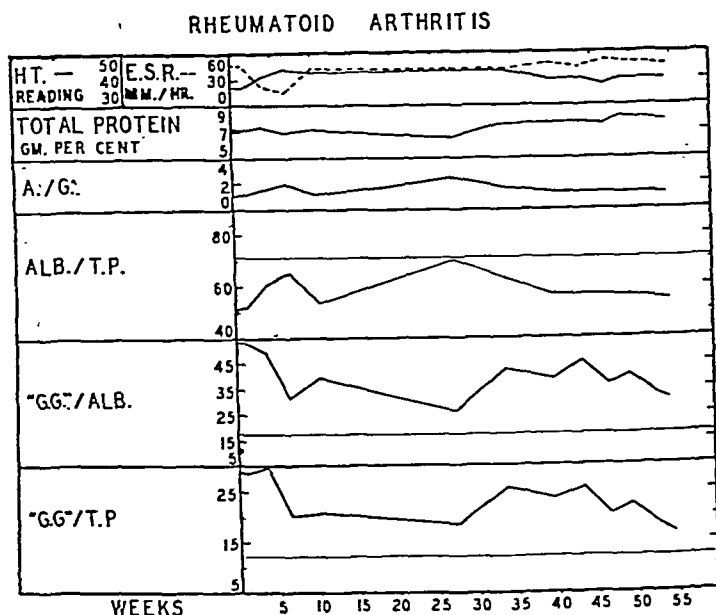


FIG. 3. IN THIS PATIENT WITH CHRONIC ARTHRITIS, ABNORMALITIES IN PROTEIN CONSTITUENTS INCREASED DURING PERIODS OF INTENSE ACTIVITY AND DIMINISHED DURING PERIODS OF RELATIVE QUIESCENCE OF THE PROCESS

ease to give attention to the ratios of various protein fractions to each other rather than the absolute values. For detecting qualitative alterations in serum protein during acute and chronic infections, the ratio of G.G. 33.3 to albumin usually gives the most striking abnormality since each component tends to change in a different direction. In certain pathologic sera as in normal sera, the albumin value, obtained with the sodium sulfate technique, may be excessively high as compared to electrophoretic measurements (4, 10, 11).

The increases in G.G. 33.3 which we have observed in such diseases as malaria, beta hemolytic streptococcal pharyngitis, acute rheumatic fever, rheumatoid arthritis, disseminated lupus erythematosus, cirrhosis of the liver, infectious hepatitis and lymphogranuloma inguinale are qualitatively similar to the increases that have been observed electrophoretically in these disorders by other investigators (4, 12 to 19). Likewise the decrease in this constituent in the nephrotic syndrome is in agreement with electrophoretic findings (10, 20).

Serial determinations of protein fractions by chemical means during specific illnesses

In a number of diseases, the electrophoretic pattern of the plasma or serum has been determined repeatedly during the course of the illness in individual cases. In Figures 1 to 4 are presented changes in protein constituents as determined by chemical measurements in a few selected cases of specific diseases. It is not pertinent at this time to discuss the clinical significance of these changes but rather to indicate that they correlate at least qualitatively with the changes in similar cases in which repeated electrophoretic measurements have been obtained.

In Figure 1 the protein changes in an uncomplicated case of streptococcal pharyngitis are shown. In this particular case the protein abnormalities persisted for a considerable period after clinical recovery had ensued and the erythrocyte sedimentation rate had become normal. This pattern is somewhat similar to that obtained by Dole and coworkers (13) in electrophoretic studies made during the course of scarlet fever.

Figure 2 illustrates the changes in protein con-

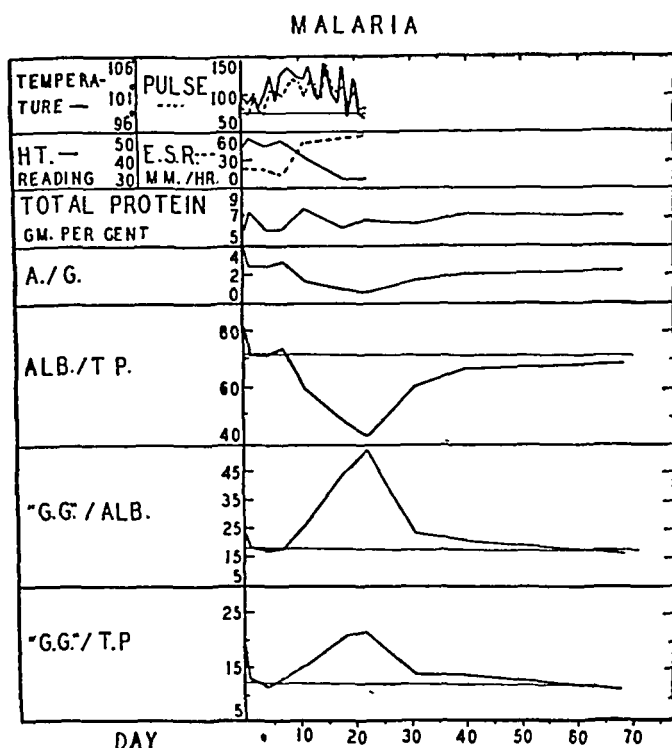


FIG. 4. THIS PATIENT WITH MALARIA INOCULATA (*PLASMODIUM VIVAX*) DEVELOPED MARKED ABNORMALITIES IN THE PLASMA PROTEINS DURING THE FEBRILE ILLNESS.

These changes slowly subsided after the fever was terminated on the 22nd day by administration of quinine.

stituents as determined chemically in a somewhat protracted case of acute rheumatic fever. In this instance, the "gamma globulin" remained elevated for a number of weeks after evidence of clinical activity had subsided and after the erythrocyte sedimentation rate had become normal. Somewhat similar observations were made in serial electrophoretic determinations of protein constituents in this disease by Dole and coworkers (13) and by Rutstein and colleagues (14).

The alterations in protein constituents in a protracted case of rheumatoid arthritis are presented in Figure 3. In this patient who was followed for one year, there were great fluctuations in the intensity of the disease as evidenced by clinical observations. Throughout most of the period of observation, the erythrocyte sedimentation rate remained greatly elevated. A rough correlation existed between clinical activity of the arthritis and the degree of abnormality in protein constituents. The changes observed in this patient are somewhat similar to the electrophoretic changes observed in an arthritic patient by Dole and Rothbard (15).

Finally, Figure 4 illustrates the changes in the protein pattern of a patient with central nervous system syphilis who was subjected to malaria inoculata (*plasmodium vivax*). Fever was terminated on the 22nd day by quinine administration. In this patient a marked hypoalbuminemia developed during the febrile illness and was accompanied by an increase in the "gamma globulin." With subsidence of the infection, the protein constituents gradually returned toward normal values. Electrophoretic studies of protein components in similar patients have demonstrated similar findings (21).

Possible clinical usefulness of gamma globulin determinations as indicated from reported electrophoretic studies

Many of the electrophoretic findings in sera of diseased subjects have been summarized in the recent paper by Stern and Reiner (22). It has been demonstrated that many antibodies to human infections are concentrated in the gamma globulin fraction (23, 24). Significant increases in gamma globulin occur during acute and chronic infections as well as in cirrhosis and congestive heart failure. Because of the numerous disorders in which this fraction is increased, its diagnostic value, as evidenced from a single determination, appears to have limited value. Even single determinations, however, in selected instances, when taken in conjunction with other clinical studies, may offer useful information. For example, in cirrhosis of the liver, the gamma globulin is greatly increased whereas in livers involved with metastatic carcinoma, this fraction is not increased (18). The finding of an elevated rather than a reduced gamma globulin content in the nephrotic syndrome suggests an acute exacerbation of the renal disease (10). The occurrence of a greatly elevated gamma globulin in a patient, suspected of psychogenic rheumatism, would cause one to consider more seriously the possibility of rheumatoid arthritis or a related disorder. Likewise, the finding of a normal gamma globulin content in the sera of patients, suspected of having certain diseases in which this fraction usually is increased, would have some value in exclusion of such processes.

The real value of serial gamma globulin deter-

minations during the course of specific illnesses remains to be learned. From serial electrophoretic studies, it would appear that the rise and fall of gamma globulin in such diseases as beta hemolytic streptococcal pharyngitis, rheumatic fever, rheumatoid arthritis, pulmonary tuberculosis and sarcoid might be a useful aid in evaluating the activity of these diseases (13 to 15, 25, 26). In tuberculous infections at least, the gamma globulin fraction appears useful in determining the prognosis (25).

SUMMARY

From an examination of 30 normal sera, the mean values were determined for total serum protein, serum albumin, serum globulin and "gamma globulin" as determined by chemical procedures. The standard deviation and coefficient of variation for these constituents are indicated.

Similar studies were made repeatedly in 5 healthy subjects in order to determine the variation in these constituents in a given individual from time to time.

Serum albumin, serum globulin and serum "gamma globulin" were measured in a number of patients with various diseases. It is demonstrated that these values show at least qualitative similarity to electrophoretic findings in the same diseases.

The possible usefulness of serial determinations of these protein constituents during the course of specific illnesses is illustrated by a few examples.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXVI. INACTIVATION OF THE VIRUS OF HOMOLOGOUS SERUM HEPATITIS IN SOLUTIONS OF NORMAL HUMAN SERUM ALBUMIN BY MEANS OF HEAT^{1,2}

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The extensive administration of whole blood, plasma, and serum in the past few years has made the problem of homologous serum hepatitis an important one. The 23,000 cases of hepatitis in Armed Forces personnel (1) resulting from the injection of certain lots of yellow fever vaccine which had been stabilized with human serum gave great impetus to study of the disease in this country.

Most epidemiological investigations suggest strongly that the risk of transmitting hepatitis is greater with plasma than with whole blood (2). This presumably depends on the practice of pooling the plasma from a number of donors, an occasional one of whom may harbor the virus, and of administering each pool to multiple recipients.

In the preparation of pooled plasma for the Armed Services from blood collected by the American Red Cross during the war, the size of the pools was set at 25 or 50 bloods depending upon final distribution in packages of 250 ml. or of 500 ml. In the preparation of normal human serum albumin, similarly collected pools of plasma representing from 250 to 2,000 bloods were used as starting material for the process of plasma frac-

tionation. The large size of these pools made it seem probable that a considerable number of them would be contaminated with the virus of homologous serum hepatitis. This was a source of great concern to those directly involved in the plasma fractionation program and to the subcommittee on Blood Substitutes of the National Research Council. Consequently, efforts were made to develop methods for the inactivation of hepatitis virus in solutions of normal human serum albumin.

The hepatitis viruses are known to be highly resistant to chemical and physical agents. They survive heating at 56° C for one hour (3), a temperature usually employed for the inactivation of viruses. They remain active for at least several years in the frozen state and withstand repeated thawing and refreezing (4). In desiccated yellow fever vaccine, the icterogenic property was still present after at least one year of storage at room temperature (5). Hepatitis viruses in plasma have remained active in the presence of merthiolate in a concentration of 1:2000 (6), equal parts of phenol and ether in a 0.5% concentration (7), tricresol in a 0.2% concentration (8), and chlorine in excess of that required to destroy the pathogenic bacteria commonly found in drinking water (9).

The remarkable thermal stability of normal human serum albumin solutions suggested the use of heat as the most likely method for the inactivation of the hepatitis virus. It was clear that the temperature required to inactivate the virus of hepatitis would be relatively high. It was therefore necessary to select conditions as rigorous as possible in order to obtain the greatest chance of destroying the virus, yet within limits which albumin solutions could withstand. The investigations

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² This paper is No. 70 in the series of studies on Plasma Proteins from the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from bloods collected by the American Red Cross.

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of Luck and his associates on stabilization (10, 11) and of Scatchard and his co-workers on the development of low-salt albumin (12) revealed that the addition of certain non-polar anions to solutions of albumin would markedly increase their thermal stability. The use of 0.04 M acetyltryptophane as a diluent resulted in an albumin solution which could be heated for 10 hours at 60° C without decreasing its stability below that of unheated standard preparations in which 0.3 M NaCl was the diluent. Consequently, this duration and degree of heating were agreed upon for trial. Since even higher temperatures could be attained by the use of larger amounts and different combinations of stabilizing reagents, it was also decided to test the effect of a 10-hour period at 64° C, using 0.02 M sodium caprylate in addition to 0.04 M acetyltryptophane to further enhance stability.

PROCEDURE

Owing to the lack of known susceptible laboratory animals, human volunteers must be inoculated in order to demonstrate the presence of active hepatitis virus in a given material. Because of the scarcity of volunteers, only limited experiments could be conducted. Plasma previously proven on repeated occasions to contain a virus of homologous serum hepatitis (5) was selected for addition to the albumin solution to be treated and tested. A 25% solution of normal human serum albumin from a single lot was prepared for this experiment, one portion containing 0.04 M acetyltryptophane, and another portion 0.04 M acetyltryptophane and 0.02 M sodium caprylate.

The icterogenic plasma which had been kept frozen at -70° C was shipped from Philadelphia to Boston on dry ice. It was thawed at 37° C and divided into 3 portions which were treated as follows:

Mixture A (control): Ten ml. of plasma were mixed with 40 ml. of 25% human serum albumin solution stabilized with 0.04 M acetyltryptophane. This mixture was stored in a tightly stoppered vaccine vial in the refrigerator until the heating of mixtures B and C was completed.

Mixture B: A mixture of 10 ml. plasma and 40 ml. of 25% albumin solution stabilized with 0.04 M acetyltryptophane was prepared in a tightly stoppered sterile vaccine vial. This was completely submerged in a water bath at 60° C for 10 hours.

Mixture C: A mixture of 10 ml. of plasma with 40 ml. of 25% albumin solution stabilized with 0.04 M acetyltryptophane and 0.02 M sodium caprylate was prepared in a tightly stoppered vaccine vial. This was completely submerged in a waterbath at 64° C for 10 hours.

Following the period of heating, Mixtures A, B, and C were promptly frozen with dry ice and shipped in the

frozen state overnight to Trenton, N. J., where they were thawed at 37° C and injected on the same day. Fifteen volunteers were divided into 3 groups of 5 men each. Group I received Mixture A, Group II Mixture B, and Group III Mixture C. Each man was given 10 ml. of the material by intramuscular injection. The following liver function studies were carried out in all the volunteers according to standard methods already published (13): urine bilirubin, urine urobilinogen, serum bilirubin, bromsulphalein retention, cephalin-cholesterol flocculation, serum colloidal gold and thymol turbidity. Urine determinations were carried out daily and serum determinations were performed once or twice weekly from a period of at least one month prior to injection until termination of the experiment 7 months after injection. If positive findings developed, all procedures were performed daily.

RESULTS

The results of the experiment are presented in Table I. Three of the five volunteers in the control group (I) which received the unheated mixture (A) of icterogenic plasma and albumin developed hepatitis, whereas none of the volunteers (Groups II and III) inoculated with the heated mixtures (B and C) showed laboratory changes suggestive of hepatitis. In Figure 1A, B, and C are given the laboratory findings of the three men in Group I who developed hepatitis but without clinical jaundice.

The experiment indicates that heating for 10 hours at either 60° C or 64° C appeared to be adequate to inactivate this hepatitis virus in human albumin solutions. The effectiveness of this temperature for shorter periods of time or the

TABLE I

Results of the inoculation of volunteers with mixtures of icterogenic plasma and human serum albumin solutions

Group	Subject	Age	Hepatitis
I (Unheated mixture)	J. H.	33	+
	D. D.	36	+
	A. P.	25	+
	E. H.	30	-
	A. D.	32	-
II (Mixture heated for 10 hours at 60° C)	L. V.	35	-
	A. T.	34	-
	R. M.	23	-
	P. J.	22	-
	H. B.	33	-
III (Mixture heated for 10 hours at 64° C)	C. R.	32	-
	W. B.	28	-
	A. M.	29	-
	E. G.	29	-
	A. C.	31	-

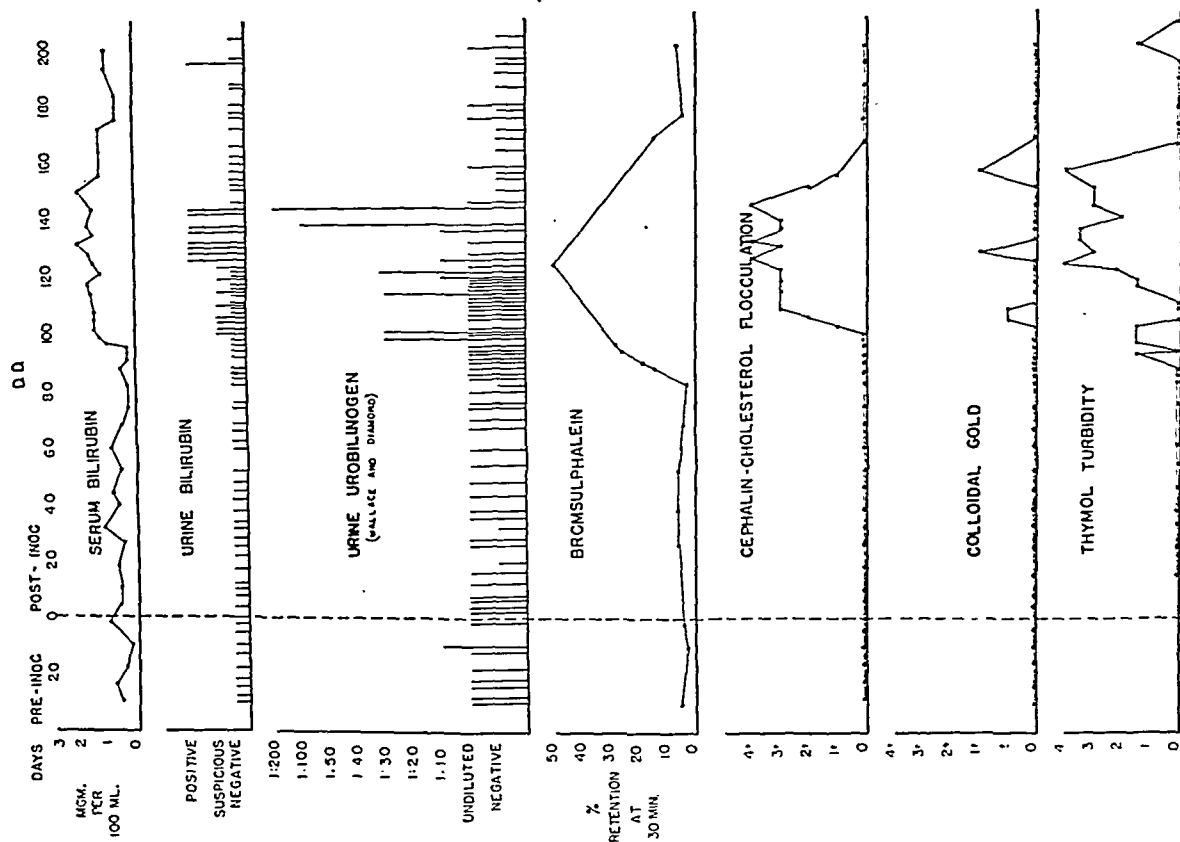


FIG. 1-B

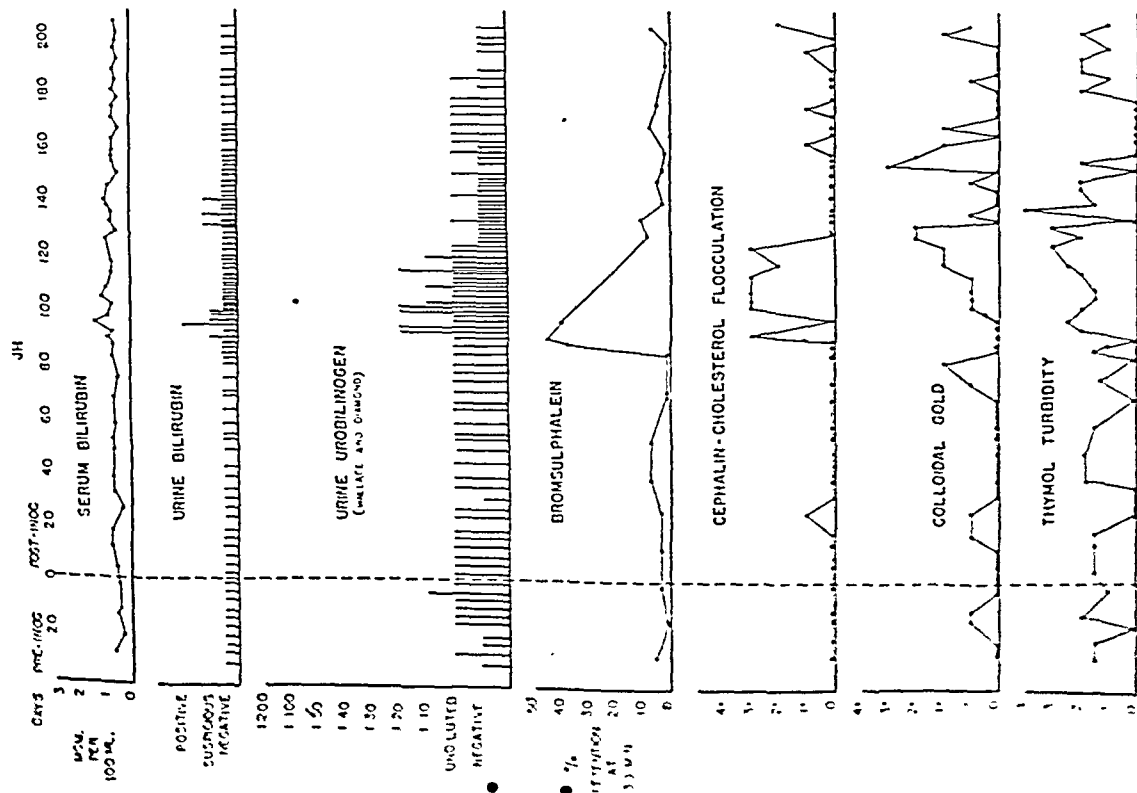


FIG. 1-A

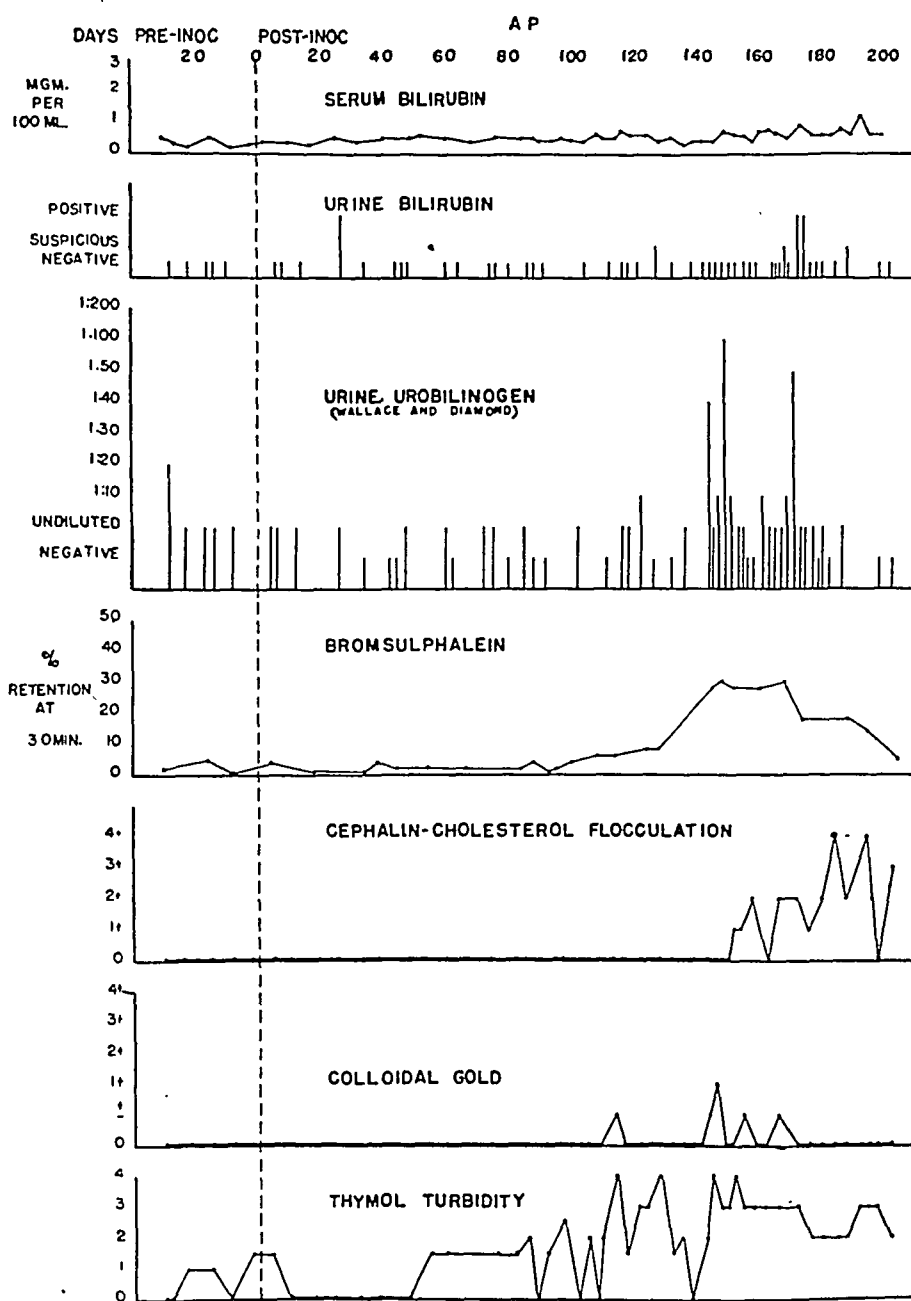


FIG. 1-C

FIG. 1 A, B, C. LABORATORY STUDIES ON THE THREE VOLUNTEERS WHO DEVELOPED LABORATORY EVIDENCE OF HEPATITIS FOLLOWING THEIR INOCULATION WITH THE UNHEATED MIXTURE OF ICTEROGENIC PLASMA AND HUMAN SERUM ALBUMIN SOLUTION

effectiveness of lower temperatures for the same period was not determined. Although the desirability of such experiments on a wider scale is evident, it was impossible to obtain larger groups of volunteers.

DISCUSSION

The need for methods of inactivating the virus of hepatitis in blood and blood products has been

emphasized by the report by Spurling *et al.* (2) who investigated the incidence of homologous serum hepatitis in recipients of 400 different pools of serum and plasma provided by one London Blood Supply Depot. Jaundice developed in 7.3% of recipients of serum and plasma, but did not occur among an equal number of recipients of whole blood.

Recent evidence makes it clear that pooled

plasma prepared from blood obtained in the United States is likewise frequently icterogenic. In a study made by the New York State Department of Health (14) the incidence of homologous serum jaundice following the administration of reconstituted dried plasma distributed by the American Red Cross has been approximately 4.5% of 649 patients on whom adequate data could be obtained. A study of the experience of a hospital blood bank, using frozen plasma, revealed at least 11 cases of serum jaundice in a year during which 949 units of pooled plasma and 1,494 units of whole blood were administered to patients (15).

These figures indicate that the fears concerning the danger of wide dissemination of homologous serum jaundice by normal human serum albumin are fully justified since the albumin was prepared from far larger pools than plasma and each lot was distributed to many more recipients. Because of these fears, studies of the effect of the plasma fractionation process on viruses were made. Bird, Enders, and Boyd (16) showed that viruses added to pooled plasma survived a small scale fractionation procedure in the laboratory. Theiler's mouse encephalomyelitis virus, vaccinia virus, tobacco mosaic virus, and tobacco necrosis virus were added to a small pool of plasma and fractionated to yield the major fractions. Titration of the viruses in the original plasma and in the fractions indicated that these viruses were neither destroyed nor concentrated in any particular fraction. Actual clinical experience has demonstrated that the use of fraction II (Gamma or immune serum globulin) in several thousand children has not been followed by hepatitis (17). Much less extensive experience with normal serum albumin has failed to reveal cases of hepatitis attributable to it. Nevertheless, the large size of the plasma pools from which plasma fractionation products are prepared, and the apparent lack of deleterious effect of the fractionation process on test viruses made imperative these studies on heat inactivation of the virus of homologous serum hepatitis in albumin solutions. Few experiments conducted up to the present time have been designed deliberately to inactivate or neutralize the hepatitis virus in whole blood, serum or plasma. Oliphant (18) has reported several studies in which a hepatitis virus in yellow fever vaccine or in serum apparently was inactivated by irradiation with ultra-

violet light. Similar studies carried out by MacCallum (19) failed to confirm fully the work of Oliphant but the method employed appears promising and certainly deserves further investigation.

Stokes and his co-workers (20, 21) have prevented infectious (epidemic) hepatitis in men exposed to the disease by intramuscular injections of gamma globulin. The effect of gamma globulin is thought to be due to the presence of protective substances in the globulin. Less conclusive results in the protection by gamma globulin against homologous serum hepatitis (22, 23) may have been due to the administration of inadequate quantities of gamma globulin. Studies in volunteers now in progress may clarify further the role of gamma globulin in the prevention of homologous serum hepatitis (24).

Heating at 60° C for 10 hours is a practical procedure in the large scale preparation of human albumin solutions, and this step now is included in their routine preparation (12). It appears probable, therefore, that human albumin solutions so prepared will be free from the risk of viral hepatitis. Unfortunately, this method cannot be applied to serum or plasma, as coagulation occurs at 60° C.

Although such heating does not seriously alter the properties of albumin solutions which have been properly stabilized it will lower the total duration of time during which the protein will withstand heating, particularly if any deviations from standard methods may have occurred during processing. Therefore, it would be desirable to know whether a shorter period of heating would regularly inactivate the virus or whether the process of fractionation itself regularly gives rise to a safe albumin solution. Unfortunately, the long incubation period of homologous serum hepatitis and the necessity for the use of human volunteers seriously handicap further studies on these questions.

SUMMARY

1. Because of the large size of the plasma pools used as starting material for the preparation of the products of plasma fractionation and because viruses added to plasma experimentally have been detected in all the major fractions obtained from it, contamination of solutions of normal human serum albumin with the virus of homologous

serum hepatitis appears possible. Accordingly methods of inactivation of this virus in serum albumin were sought.

2. On the basis of limited experiments the virus of homologous serum hepatitis appeared to be inactivated when stabilized human albumin solutions to which the virus was added were heated at 60° C and 64° C for 10 hours. This degree and duration of heating apparently do not seriously alter the measurable chemical or physical properties of the albumin.

3. The minimum amount of heating required to inactivate this virus was not determined.

4. The method described is not applicable to whole blood, plasma, or serum.

5. Heat treatment at 60° C for 10 hours in the final container is now a routine step in the preparation of human albumin solutions. Clinical use of albumin so prepared would appear to be free from the risk of transmission of active serum hepatitis virus.

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THE ANEMIA OF INFECTION. VII. THE SIGNIFICANCE OF FREE ERYTHROCYTE PROTOPORPHYRIN, TOGETHER WITH SOME OBSERVATIONS ON THE MEANING OF THE "EASILY SPLIT-OFF" IRON¹

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Studies in this laboratory (1) on the anemia accompanying infection have indicated that there is associated with such anemia a disturbance in the metabolism of porphyrins. The amount of free protoporphyrin in the erythrocytes has been found to be increased and the excretion of coproporphyrin in the urine is greater than normal. The present report deals with studies designed to investigate the significance of the free protoporphyrin in the erythrocytes.

The presence of free protoporphyrin in the erythrocytes (EP) was reported in 1928 by van den Bergh and Hyman (2). Grotepass (3) and subsequently Watson, Grinstein and Hawkinson (4) demonstrated that the free protoporphyrin in erythrocytes is identical with the protoporphyrin in hemoglobin; namely, protoporphyrin 9, type III. Seggel (5) concluded that the protoporphyrin resides in erythrocytes which exhibit red fluorescence in ultraviolet light. He named such cells fluorescytes and regarded them as being either reticulocytes or adult erythrocytes containing porphyrin. Seggel suggested that free protoporphyrin represents an intermediate compound in the synthesis of hemoglobin. He presented evidence that the fluorescytes are increased as a result of iron deficiency or as a consequence of other disturbances of hemoglobin synthesis (5).

Watson and Clarke (6) observed upon centrifuging different samples of blood that the upper layer of the packed cells, which is richer in reticulocytes, showed a much higher content of proto-

porphyrin than the lower layer. They concluded that the protoporphyrin resides mainly in the reticulocytes. Similar conclusions were reached later by DeLangen and Grotepass (7). Further studies by Watson and his associates (4, 8) showed, however, that although there is frequently a close parallelism between the increase in protoporphyrin and normoblastic activity, as measured by the percentage of the reticulocytes, several other factors influence the protoporphyrin content of the erythrocytes. These are (a) the presence of iron deficiency or of factors interfering with the utilization of iron in the synthesis of hemoglobin as, for example, lead poisoning; and (b) the presence of conditions which permit the formation of protoporphyrin by degradation of hemoglobin in intact erythrocytes. The formation of protoporphyrin from hemoglobin in intact erythrocytes would be a simple explanation of the increase observed after incubation of blood *in vitro* (4). Cziike (9), Barkan and Walker (10), and Watson and Paine (11) have presented evidence indicating that hemoglobin breakdown can take place inside the intact red cell. According to the last two groups of workers, this is through the formation of *pseudohemoglobin* (Barkan) or *verdohemoglobin* (Lemberg). From studies mainly carried out by Barkan and Schales (12) and Lemberg *et al.* (13, 14) it would seem that the non-hemoglobin iron of the erythrocytes which is easily ionized by the incubation of blood in 0.4 per cent HCl for 16 to 24 hours is part of a *bile pigment-iron-native globin* complex similar to or identical with the pseudohemoglobin of Barkan or the verdohemoglobin of Lemberg. According to Barkan this iron, known as "easily split-off iron" (ESFe), corresponds to about 5 per cent of the total hemoglobin iron found in human beings. Lemberg (13) claims that two-thirds of the ESFe is an artefact due to the oxidation of the

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prosthetic group of the hemoglobin by the oxygen evolved from oxyhemoglobin by acids.

An increase in free erythrocyte protoporphyrin has been found in cases associated with hemolytic anemia (8). In such cases one would wonder whether some of the increase may be due to protoporphyrin formed in the cells as a degradation product. Nevertheless, since in most instances of hemolytic anemia an increase in reticulocytes occurs, the possibility arises that the increase in EP is related to a high percentage of young cells rather than due to the degradation that may take place in the mature cells.

In an attempt to seek further information concerning the significance of EP, it was decided to measure the free EP of erythrocytes under various experimental conditions and to calculate the average free protoporphyrin of mature cells and reticulocytes. At the same time, since the results of several authors (15 to 17) raise the question whether ESFe is a labile hemoglobin iron or just an artefact, the opportunity was taken in the experiments presented here to secure some data which might throw light on the significance of the ESFe.

METHODS AND CALCULATIONS

When the blood is centrifuged the reticulocytes, being lighter than mature cells, remain in the upper part of the packed cells (18). In order to secure concentrated specimens of reticulocytes and of mature cells, after separating the plasma, the column of packed cells of various samples of blood was divided into three equal parts: the upper (u) and lower (l) parts were then suspended separately in 0.9 per cent NaCl to approximate the original volume. In both fractions the reticulocyte percentage was determined by the brilliant cresyl blue wet stain method (19). The middle portion was discarded. We have found (Table I) that in suspending rabbits' cells

TABLE I

Showing that suspending red cells in 0.9 per cent saline solution has no effect on the mean corpuscular volume (MCV)

Whole blood			After suspension in 0.9% NaCl soln.		
RBC	Ht	MCV	RBC	Ht	MCV
4.19	34.0	81	4.26	34.4	81
4.13	35.1	83	4.45	37.0	82
4.31	34.0	79	4.45	33.5	76
4.52	34.0	75	4.57	33.5	73
5.56	41.0	74	5.10	41.5	81
5.03	35.0	69	4.99	36.0	72

in 0.9 per cent saline, the mean corpuscular volume (MCV) remains unchanged. By determining the percentage of the reticulocytes and mature cells in each fraction, the following three systems of equations could be established:

$$1. \frac{100 - a}{100} X + \frac{a}{100} Y = \text{MCV of the upper fraction}$$

$$\frac{100 - b}{100} X + \frac{b}{100} Y = \text{MCV of the lower fraction,}$$

when

a is the reticulocyte percentage of the upper fraction;
 b is the reticulocyte percentage of the lower fraction;
 X is the mean corpuscular volume of the mature erythrocytes (MCV_E); and

Y is the mean corpuscular volume of the reticulocytes (MCV_R).

$$2. \frac{100 - a}{100} X + \frac{a}{100} Y = P_u$$

$$\frac{100 - b}{100} X + \frac{b}{100} Y = P_l, \text{ when}$$

P_u is the free protoporphyrin of the upper fraction expressed in micrograms per 1,000 ml. of packed cells;

P_l is the free protoporphyrin of the lower fraction expressed in micrograms per 1,000 ml. of packed cells;

X is the free protoporphyrin per 1,000 ml. of packed mature erythrocytes expressed in micrograms (P_E); and

Y is the free protoporphyrin per 1,000 ml. of packed reticulocytes expressed in micrograms (P_R).

If the concentration of the protoporphyrin per 1,000 ml. ($10^{15} \mu^3$) of packed mature cells and reticulocytes is known, the mean content of free erythrocyte protoporphyrin in the mature red cells (MEP) and the mean reticulocyte free protoporphyrin (MRP) can be calculated by the following equations:

$$\text{MEP} = \frac{P_E \times \text{MCV}_E}{10^{15}}$$

$$\text{MRP} = \frac{P_R \times \text{MCV}_R}{10^{15}}.$$

The values for MEP and MRP are expressed in micro-micrograms [(10^{-15}) gm.].

$$3. \frac{100 - a}{100} X + \frac{a}{100} Y = \text{ESFe}_u \text{ (per cent)}$$

$$\frac{100 - b}{100} X + \frac{b}{100} Y = \text{ESFe}_l \text{ (per cent), when}$$

ESFe_u is the "easily split-off" iron of the upper fraction expressed in proportion to the total hemoglobin iron;

ESFe_l is the "easily split-off iron of the lower fraction expressed in proportion to the total hemoglobin iron;

X is the "easily split-off" iron of the mature erythrocytes expressed in relation to the total hemoglobin iron (ESFe_E);

Y is the "easily split-off" iron of the reticulocytes expressed in relation to the total hemoglobin iron (ESFe_R).

The following determinations were run on the whole (w) blood as well as on the upper (u) and lower (l) fractions: red blood cell counts using the bright line Spencer counting chamber and a standardized red cell pipette; volume of packed cells, using the Wintrobe hematocrit tube; hemoglobin, by the photoelectric oxyhemoglobin method, using an Evelyn photoelectric colorimeter. The instrument was standardized by the Van Slyke procedure as well as by the hemin method of Clegg and King (20). The fragility of the red cells was measured by the photoelectric method of Hunter (21). The number of determinations was limited, however, by using only 0.78 per cent saline for dilution of the blood and measuring the hemoglobin concentration of the supernatant fluid.

Plasma iron was determined by the method of Barkan and Walker (22) but twice the quantities recommended by them were used. Plasma bilirubin was determined by the method of Ducci and Watson (23). "Easily split-off" iron was measured by the method of Barkan and Walker (22) with the following modification: one ml. of whole blood was centrifuged, the plasma removed and the cells hemolyzed with iron-free water, the volume being finally made up to 5 ml. One ml. of the upper and lower fractions of the blood was treated in exactly the same way and made up to a volume of 5 ml. It was decided not to centrifuge and discard the supernatant fluid because this would permit loss of a significant frac-

tion of the ESFe, since Barkan (24) showed that the more fragile cells contain more ESFe. To the 5 ml. of hemolyzed cells, 2.5 ml. of 1.2 per cent of HCl was added and incubated for 16 to 24 hours. The remaining steps of the procedure as outlined by Barkan were then followed without modification.

Free erythrocyte protoporphyrin (EP) was determined by a modification of the method of Grinstein and Watson (25) measuring the absorption exactly at the wave length of maximum absorption (411 $m\mu$) of the protoporphyrin in 25 per cent HCl. The Beckman spectrophotometer was used. With this apparatus the sensitivity of the method is increased about five times. The details of the modified method requiring only 2 to 3 ml. of blood will be described elsewhere (26).

The measurements which have been described and the calculations derived therefrom were carried out in the following experiments.

1. Phenylhydrazine hemolytic anemia in rabbits:

Hemolytic anemia was produced in rabbits by injecting intraperitoneally a 5 per cent solution of phenylhydrazine hydrochloride. According to the size and age of the rabbits, from 50 to 150 mgm. were injected. Blood samples were taken by cardiac puncture at appropriate intervals before and after the drug was given.

TABLE II
Changes observed in a rabbit following injection of phenylhydrazine

Date	Blood*	RBC	Hb	Ht	MCV	MCH	MCHC	Retic.	EP	Bil.	Pl. Fe.	ESFe
		mill./c. mm.	gm./100 ml.	ml./100 ml.	c. μ .	$\gamma\gamma$	%	%	$\gamma/100$ ml. RBC	mgm.	$\gamma/100$ ml. pl.	% of Hb iron
1/27	w	6.10	13.95	40.8	67	23	34	4.6	63	0.19	178	3.55
1/27	100 mgm. of phenylhydrazine HCl injected intraperitoneally											
1/28	w	3.90	10.39	28.0	72	27	37	28.9	205	0.16	242	4.90
	u	3.65	11.40	38.0	85	31	37	43.5	259	—	—	4.66
	l	3.55	8.38	22.0	62	24	38	12.6	208	—	—	5.88
1/29	w	2.59	6.90	19.5	75	27	35	39.4	230	0.16	317	3.91
1/30	w	2.31	6.41	19.0	82	28	34	45.6	485	0.16	362	3.58
	u	2.19	6.06	21.0	96	28	29	63.4	746	—	—	2.81
	l	2.73	7.84	20.0	73	30	39	6.8	237	—	—	4.35
1/31	w	2.03	5.48	18.2	91	27	30	57.2	205	0.13	530	3.13
2/3	w	3.14	8.71	33.0	105	28	26	42.4	253	0.13	143	2.74
	u	3.22	8.64	36.2	112	27	24	60.1	294	—	—	3.07
	l	3.41	7.55	29.1	85	22	26	23.2	207	—	—	3.68
2/7	w	4.30	10.27	37.8	88	24	27	18.6	98	0.12	217	3.52
	u	4.42	10.52	40.5	92	24	26	46.6	109	—	—	3.98
	l	3.97	9.57	33.3	84	24	28	11.6	92	—	—	3.60
Calculated data:		Date	MCV _x	MCV _m	P _x	P _m	MEP	MPP				
		1/28	53	127	188	352	100	447				
		1/30	70	111	177	1007	124	1190				
		2/3	68	141	152	388	103	547				
		2/7	82	100	87	131	71	131				

* w refers to whole blood; u, to upper part; l, to lowest part.

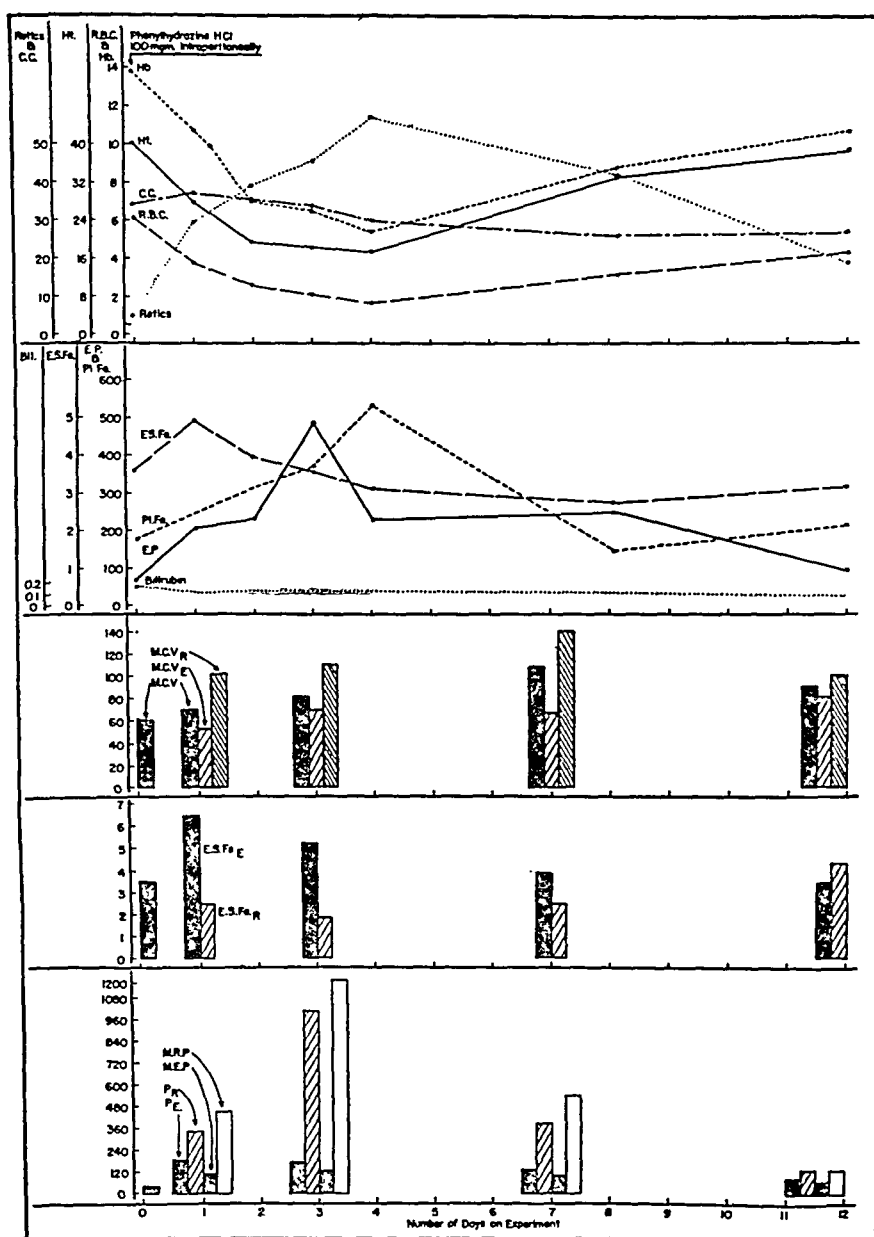


FIG. 1. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE BLOOD OF A RABBIT FOLLOWING THE ADMINISTRATION OF PHENYLHYDRAZINE

Note the parallel rise of the free erythrocyte protoporphyrin (EP) and the reticulocytes following the administration of the drug and the fact that the peak of the EP rise preceded by one day the peak of the reticulocyte curve. The ESFe ("easily split-off" iron) rose within the first 24 hours and decreased at the time of maximal reticulocytosis. The content of EP in the mature red corpuscles (P_E, MEP) showed practically no change. The quantity in the reticulocytes (P_R and MRP) was high and was greater in the reticulocytes appearing early than in those examined later. The amount of "easily split-off" iron in the mature red corpuscles (ESFe_E) was greater than in the reticulocytes (ESFe_R) except in one instance.

Explanation of Abbreviations: Hb refers to hemoglobin in grams per 100 ml.; Ht, volume of packed red cells in ml. per 100 ml.; CC, mean corpuscular hemoglobin concentration in per cent; RBC, red cell count in millions per c.mm.; Retics, reticulocytes in per cent; ESFe, "easily split-off" iron in mgm. per 100 mgm. of hemoglobin iron, expressed as per cent; Pl.Fe. refers to plasma iron in micrograms per 100 ml. of plasma; EP, the free erythrocyte protoporphyrin in micrograms per 100 ml. of packed cells; bilirubin refers to plasma bilirubin in mgm. per 100 ml. of plasma; MCV is the mean corpuscular volume of the red cells in cubic microns; MCV_E is the mean corpuscular volume of the mature erythrocytes; MCV_R is the mean corpuscular volume of the reticulocytes; ESFe_E is the "easily split-off" iron content of the mature erythrocytes; ESFe_R is the "easily split-off" iron content of the reticulocytes; P_E is the micrograms of protoporphyrin in 100 ml. of packed mature erythrocytes; P_R is the micrograms of protoporphyrin in 100 ml. of packed reticulocytes; MEP is the mean mature erythrocyte protoporphyrin expressed in micro-micrograms ($\mu\mu\text{g.}$); MRP is the mean reticulocyte protoporphyrin ($\mu\mu\text{g.}$).

2. Hemolytic anemia in sheep produced by the use of hemolysins:

Anti-sheep cell hemolysin was prepared by injecting washed sheep cell suspension (about 10 per cent saline suspension) intravenously into rabbits. This was done in progressively increasing amounts ranging from 0.5 to 2 ml., given every other day for a period of 30 days. At the end of this period, the rabbits' serum showed a titer of 1:500 against sheep's cells. Two sheep were used in this experiment.

3. Pyridoxine deficiency anemia in pigs:

This was produced as described by Wintrobe *et al.* (27).

4. Pernicious anemia:

The patient studied in this experiment was a man, 69 years of age, in relapse who was given pteroylglutamic acid in the amount of 50 mgm. orally per day.

5. Splenic stasis:

In this experiment three dogs and three pigs were used. The animals were kept under nembutal anesthesia

for four hours, after which the splenic vein and artery were exposed. The spleen was seen to be greatly enlarged. A blood sample was obtained from the splenic artery and then epinephrine hydrochloride was injected directly into the organ. As soon as the spleen started to contract, a blood sample was obtained from the splenic vein. The injection of epinephrine was observed to produce a reduction in the size of the spleen to a third of its size prior to administration of the drug.

RESULTS

1. Phenylhydrazine anemia:

In five experiments anemia was produced in rabbits by injecting phenylhydrazine. In Table II and Figure 1 are shown the results of one of these experiments. Anemia developed rapidly following the injection of 100 mgm. of phenylhydrazine hydrochloride and reticulocytes rose promptly to reach a peak on the fourth day. The total free erythrocyte protoporphyrin (EP) began to rise promptly also but the peak was reached a day

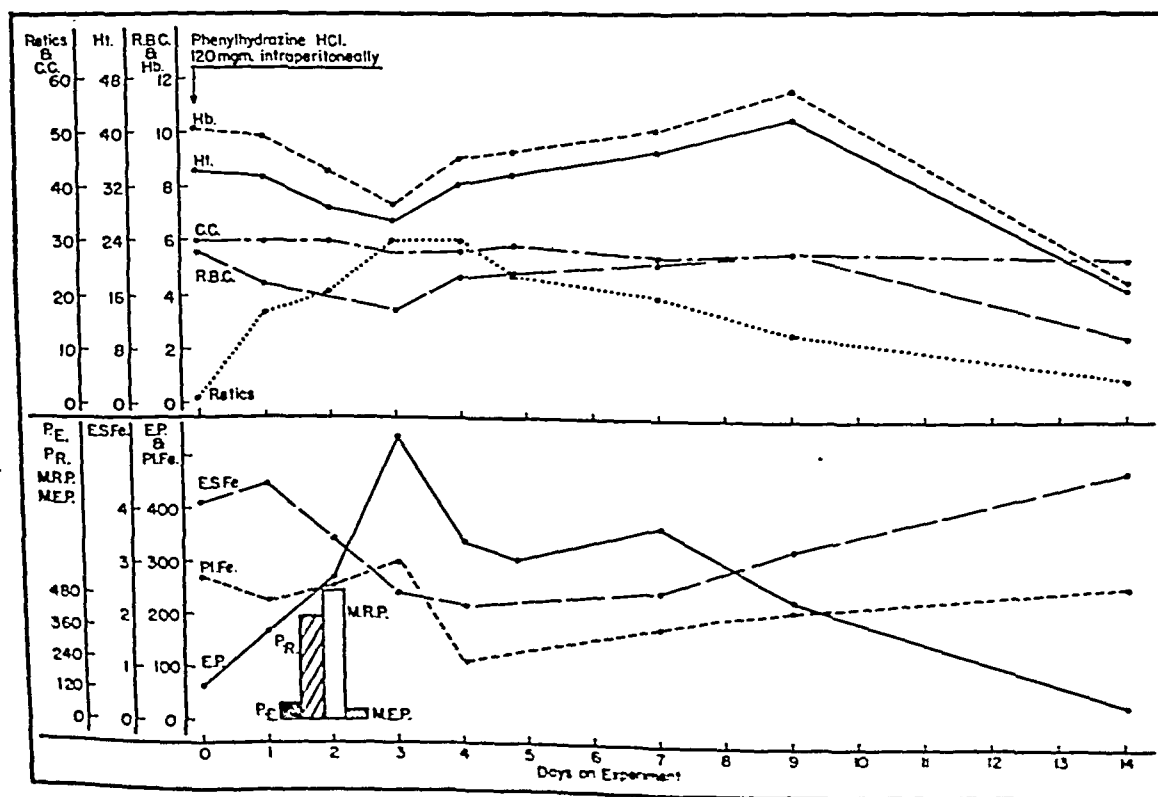


FIG. 2. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE BLOOD OF ANOTHER RABBIT FOLLOWING THE ADMINISTRATION OF PHENYLHYDRAZINE

Note that the changes were similar to those shown in Figure 1, except that the peak of the EP rise was reached at the same time as the peak of the reticulocytosis.

earlier. The "easily split-off" iron (ESFe) rose to its highest level in 24 hours. Plasma iron increased more slowly, reaching a peak on the fourth day when the anemia was most severe. As the anemia cleared, the various values returned to normal. No significant change in plasma bilirubin was observed.

It will be noted that the mean corpuscular volume (MCV) of all the cells increased, but when the size of the mature and younger (reticulated) corpuscles was calculated, the increase was seen to be due chiefly to a great increase in the size of the reticulocytes (MCV_R). Calculations of the EP content of the mature cells and the reticulo-

cytes showed little change in the amount present in the former (P_E) but a great increase in the amount found in the reticulocytes (P_R). When these values are expressed per cell, the difference becomes more striking. Thus the mean erythrocyte protoporphyrin (MEP) ranged from 71 to 124 micro-micrograms whereas the content in the reticulocytes (MRP) rose from 447 to 1,190 $\mu\mu\mu\text{g.}$, and then decreased to 131 $\mu\mu\mu\text{g.}$ In contrast with the findings just described, the quantity of "easily split-off" iron in the mature cells ($ESFe_E$) was much higher than in the reticulocytes ($ESFe_R$) except in one determination. It is noteworthy that the total ESFe of the red

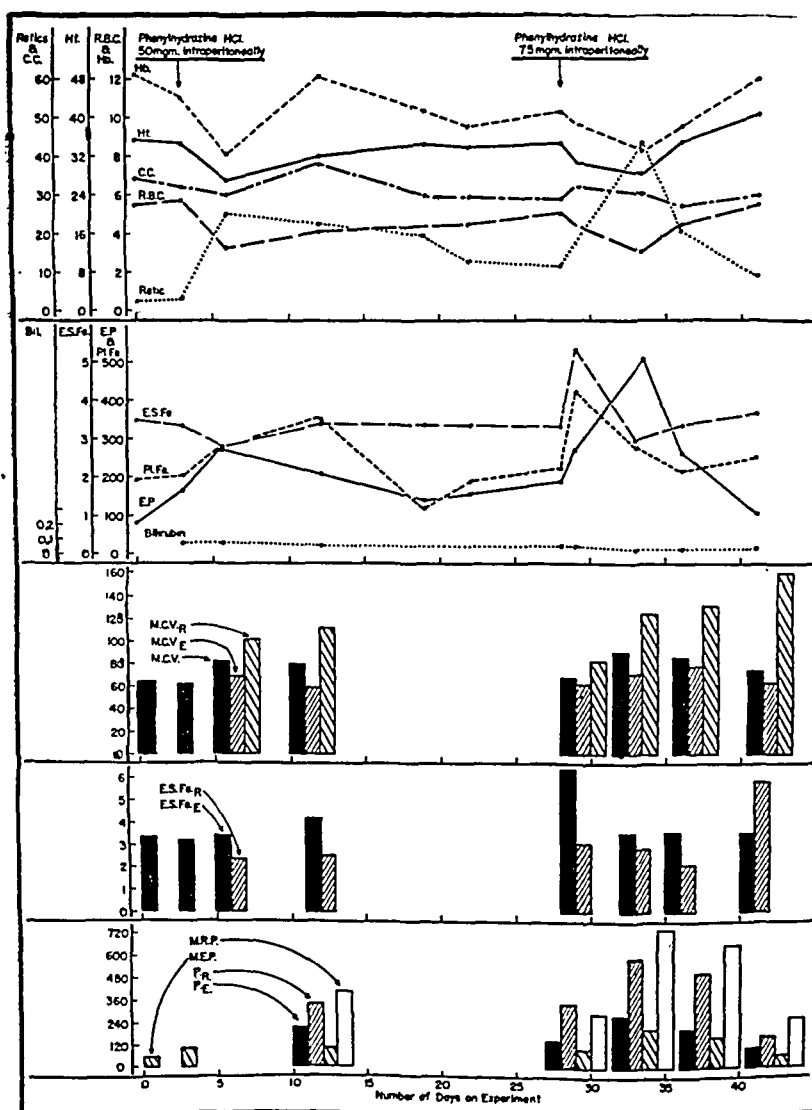


FIG. 3. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE POOLED BLOOD OF THREE RABBITS, FOLLOWING TWO SUCCESSIVE DOSES OF PHENYLHYDRAZINE

Note that the changes were similar to those shown in Figures 1 and 2.

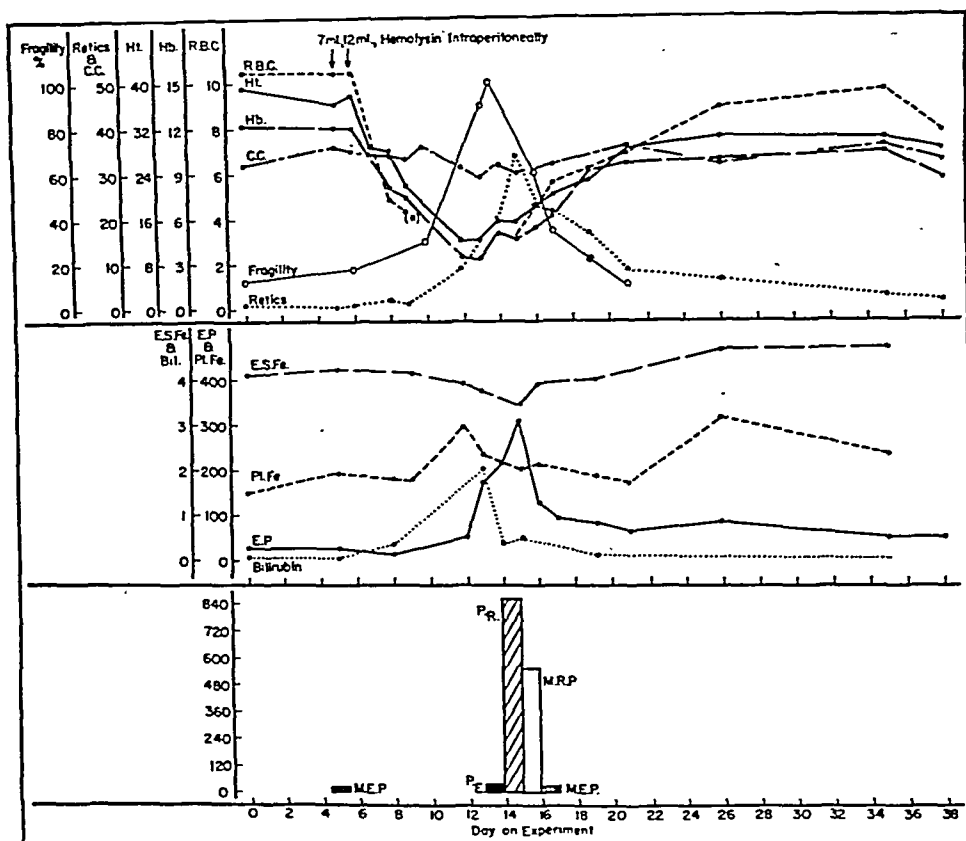


FIG. 4. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE BLOOD OF A SHEEP FOLLOWING THE ADMINISTRATION OF ANTI-SHEEP RED CELL HEMOLYSIN

Note that the EP did not rise during the period of greatest blood destruction but started to increase when the percentage of reticulocytes began to rise. The increase in EP and in reticulocytes were parallel, reaching a peak at the same time. The ESFe decreased at the time of maximal reticulocytosis. The MEP and P_R showed no increase but the MRP and P_R were very high. Plasma bilirubin showed a marked increase at the time of greatest red cell fragility. The PlFe increased as blood destruction increased and rose again later.

Fragility is expressed in per cent, this representing the amount of hemoglobin in the supernatant fluid in relation to the amount in the blood prior to dilution with hypotonic saline.

cells decreased sharply prior to the peak of the reticulocytosis.

Figure 2 presents the observations in another rabbit. These were similar to those just described except that the maximal increase in total red cell EP coincided with the maximal increase in reticulocytes. It is of interest that in this animal anemia developed spontaneously after recovery had taken place. Figure 3 presents the observations in three rabbits whose blood was pooled for the various determinations. Phenylhydrazine was given these animals in two different doses, 50 mgm. and 75 mgm., respectively, the latter having been given 30 days after the former. The changes which were noted were similar to those already

described. Thus the total red cell EP reached a maximum at the same time as the maximal reticulocyte response. ESFe increased sharply when 75 mgm. of phenylhydrazine were given, simultaneously with a rise in plasma iron, and then decreased prior to the rise in reticulocytes. Similar results were obtained in two more experiments but these were not completed because of the early death of the animals.

2. Hemolytic anemia in sheep:

The data obtained in sheep No. 1 are shown in Figure 4. Two doses (7 and 12 ml.) of anti-sheep cell hemolysin were given intraperitoneally. Anemia developed very rapidly, reaching the most

severe stage on the seventh day. At this time the red blood cells were so agglutinated that a count could not be done with Hayem's solution. This difficulty was overcome by using Gower's solution (19). The hypotonic saline fragility of the erythrocytes was maximal at the time when the anemia was most severe and the plasma bilirubin increased simultaneously. An increase of reticulocytes commenced on the fourth day, when the anemia was already quite severe. The reticulocytosis reached its peak at the tenth day after the first injection of hemolysin. It will be noted that the EP did not rise; in fact it decreased slightly at the time of the greatest red cell degradation. As in the phenylhydrazine anemia, however, the EP rose when the reticulocytes increased, the peak corresponding to the highest reticulocytosis. The only significant change in the ESFe was a

drop in this value at the time of the peak of the reticulocytosis. The plasma iron first showed an increase coinciding with the time of most severe anemia and greatest blood destruction as indicated by increased red cell fragility and plasma bilirubinemia. A second rise in plasma iron developed at the time when the blood was approaching normal.

From the calculated data, it may be seen that the MEP and P_E did not show a significant change at the time of the greatest red cell destruction. The MRP and P_R , on the other hand, were very high as was found in phenylhydrazine anemia.

The results of the experiment in sheep No. 2 are shown in Figure 5. This animal was given 5 ml. anti-sheep cell rabbit serum intraperitoneally on three successive days. It may be seen that the results are similar to those in sheep No. 1 except

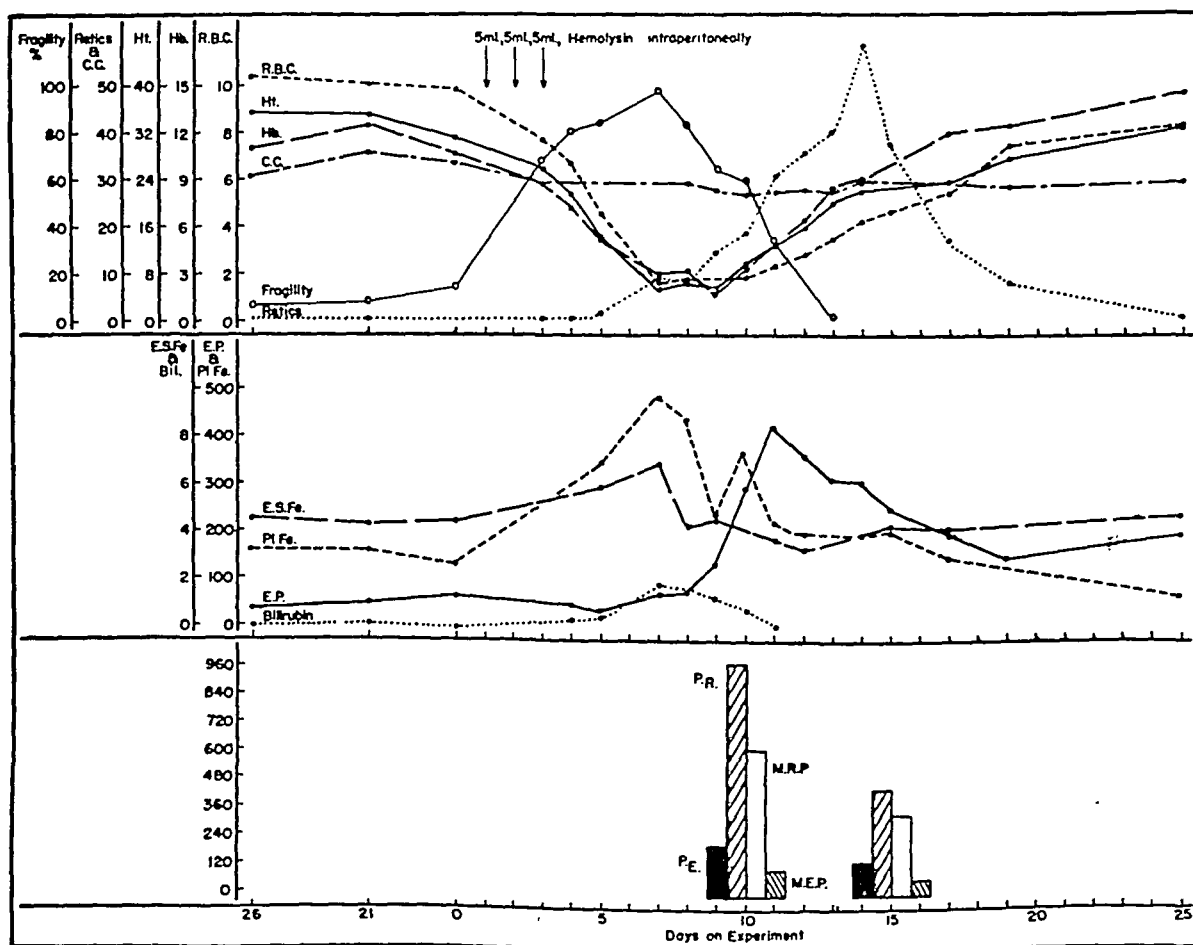


FIG. 5. SHOWING THE CHANGES IN THE BLOOD OF ANOTHER SHEEP FOLLOWING THE ADMINISTRATION OF ANTI-SHEEP RED CELL HEMOLYSIN

Note that the changes which followed were like those shown in Figure 4 except that the peak of the rise in EP preceded by one day the peak of the increase in reticulocytes and the ESFe increased at the time of the greatest red blood cell destruction.

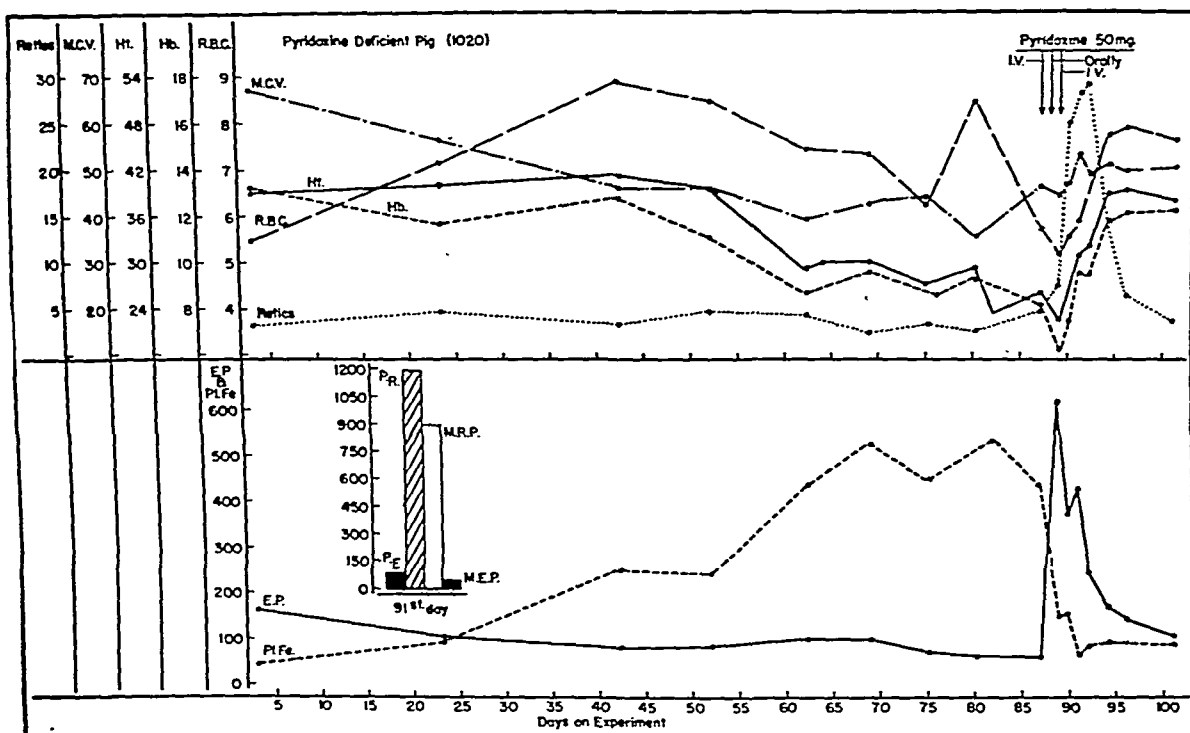


FIG. 6. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE BLOOD OF A PYRIDOXINE-DEFICIENT PIG AND THE EFFECTS PRODUCED BY ADMINISTRATION OF PYRIDOXINE

The anemia started to develop about the 52nd day following the commencement of the pyridoxine-deficient diet. This was accompanied by a decrease in the volume of the red corpuscles (MCV) and in EP as well as a great increase in the plasma iron. After treatment, there was a very marked rise in EP 48 hours following the first dose of pyridoxine which coincided with a rise in the percentage of reticulocytes, a sharp drop in the plasma iron content and an increase in the size of the red blood cells.

for the following: the peak of the EP curve was reached earlier than the peak of the reticulocyte curve and the EP persisted at an unusually high value in spite of a marked fall in the reticulocyte percentage.

3. Pyridoxine deficiency anemia in pigs:

Pyridoxine deficiency anemia developed in two pigs, 10-20 and 10-21. The data obtained in pig 10-20 are shown in Figure 6. It may be seen that anemia started to develop on the 52nd day following commencement of the pyridoxine-deficient diet. This was accompanied by a drop in the MCV, an increase in the plasma iron and a decrease in EP to an abnormally low level. The decrease in EP in pyridoxine deficiency confirms earlier observations in this laboratory (28).

These changes were most pronounced on the 82nd day after the start of the diet. After treatment with pyridoxine the reticulocytes increased

sharply, reaching a maximal value on the fifth day after the first injection was given. This was followed by an increase in the volume of packed red cells and in the size of red corpuscles. A striking change took place in the EP level. On the second day after 50 mgm. pyridoxine was given intravenously, the EP increased from 60 to 612 $\mu\text{g. per 100 ml. red cells}$. The normal value for EP in the pig is approximately 100 $\mu\text{g.}$ The EP level then dropped in spite of an increase in the reticulocyte percentage. The plasma iron dropped also on the second day after treatment to a normal level and continued at that level.

The calculated data for protoporphyrin in the reticulocytes and mature cells (MRP, MEP, P_R , P_E) in this animal showed much higher values in the immature cells, just as was observed in the experimental anemias already described.

Pig 10-21 developed anemia with similar chemical abnormalities. Although the anemia was not

TABLE III
Pyridoxine-deficient pig (10-21)

Date	RBC	Hb	Ht	MCV	MCH	MCHC	Retic.	EP	Pl. Fe.	ESFe
	mill./c. mm.	gm./100 ml.	ml./100 ml.	c. μ .	$\gamma\gamma$	%	%	$\gamma/100$ ml. RBC	$\gamma/100$ ml. pl.	% of Hb iron
3/25	6.38	13.8	42.5	67	22	32	4.0	159	89	3.76
4/17	7.50	12.6	41.0	55	17	31	5.4	105	173	4.14
5/6	9.30	13.5	44.0	47	14	31	3.6	70	268	3.80
5/16	9.50	12.3	41.0	43	13	30	4.1	51	227	3.94
5/27	8.30	11.2	35.1	42	14	32	3.2	69	360	4.64
6/5	8.80	11.7	36.2	41	13	31	3.2	77	409	4.34
6/7			38.0							
6/12	8.25	10.0	32.0	39	13	34	3.6	55	496	
6/18	8.30	11.0	35.1	42	13	31	3.8	55	462	
6/23	9.10	11.1	36.0	40	12	31	3.2	51	483	
6/25			34.0					54	454	
7/5	7.40	8.7	29.5	40	12	29	4.1	50	466	
7/7	6.80	9.1	30.0	44	13	30	2.3	50	508	
7/8	7.30	9.2	29.0	40	12	31	3.4	44	315	
7/8				50 mgm. pyridoxine given intravenously.						
7/9	10.30	13.9	46.0	45	14	30	8.6	141		
7/10				Pig died.						

as severe as in 10-20, the chemical changes were even more pronounced (Table III). The deficiency in this animal was so severe that, in spite of treatment, the animal died.

4. Pernicious anemia:

The results in a case of pernicious anemia treated with pteroylglutamic acid are shown in Figure 7. The peak of the rise in EP was reached

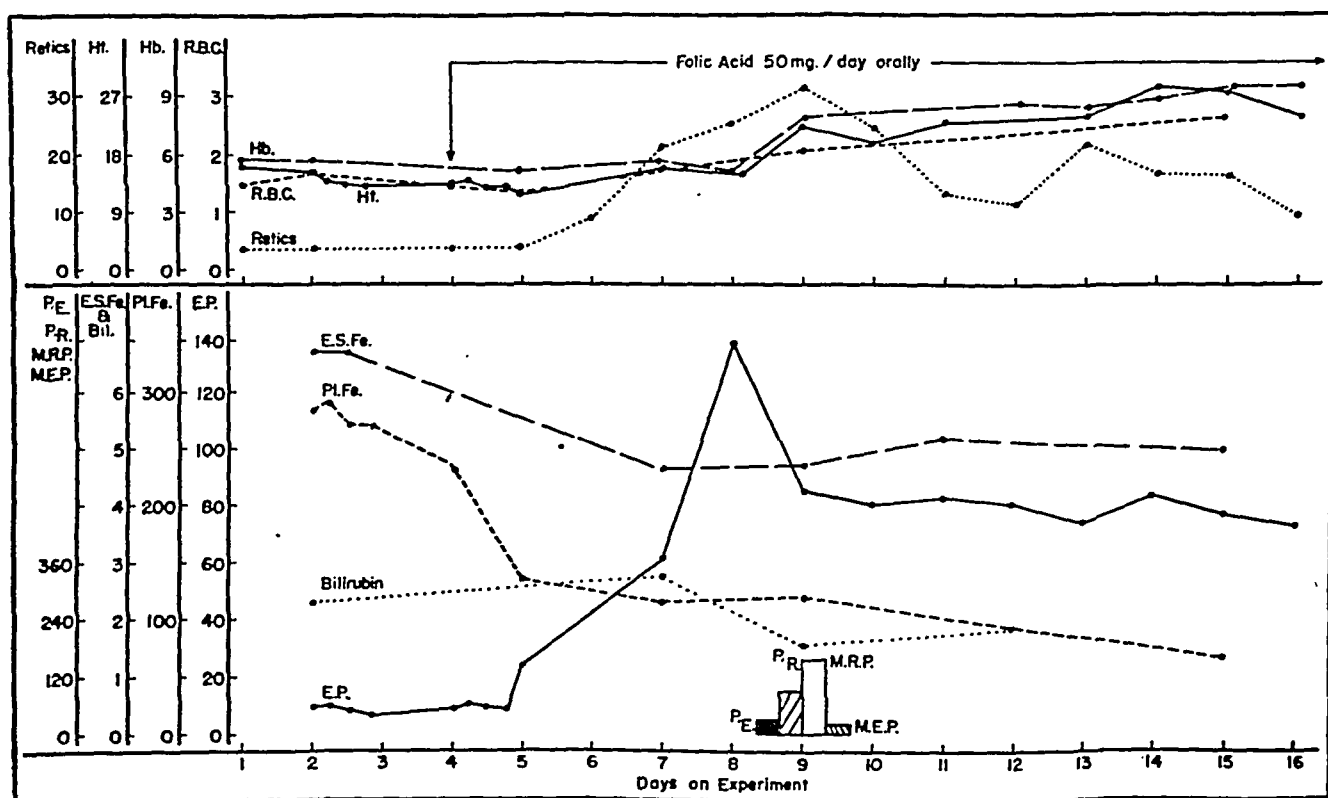


FIG. 7. SHOWING THE HEMATOLOGICAL AND CHEMICAL CHANGES IN THE BLOOD OF A PATIENT WITH PERNICIOUS ANEMIA IN RELAPSE, FOLLOWING TREATMENT WITH PTEROYLGLUTAMIC ACID

Note the consistently low EP at different hours of the day prior to therapy and the increase after treatment which coincided with the rise of the reticulocyte percentage; the peak of the EP level preceded by one day the peak of the reticulocytosis. Pl.Fe, ESFe, and bilirubin dropped after treatment. P_R and MRP were very much higher than the P_E and MEP.

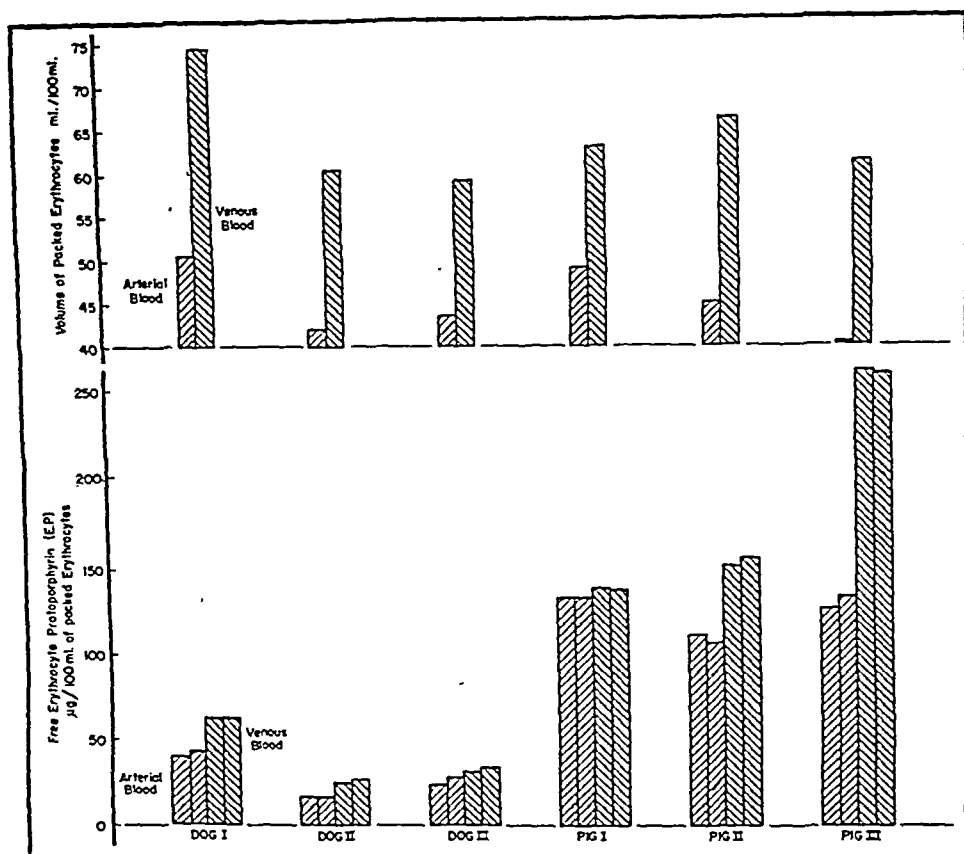


FIG. 8. SHOWING THE CONCENTRATION OF THE VENOUS BLOOD FROM THE SPLEEN AFTER STASIS DURING NEMBUTAL ANESTHESIA IN THREE DOGS AND THREE PIGS, AND THE INCREASE IN EP ASSOCIATED WITH STASIS

The EP determinations were done in duplicate and are so shown.

one day earlier than the peak of the reticulocyte curve. The ESFe dropped as the reticulocytes increased. Plasma iron and bilirubin decreased at the same time. The MEP and P_E showed no significant change after treatment; the MRP and P_R were high, just as had been found in the experimentally induced hemolytic anemias. The very low values for EP during relapse are of interest. These were observed to be constantly low during the different hours of the day.

5. Splenic stasis:

The effect of stasis was studied in order to see whether the increased EP observed by Watson *et al.* (4) during *in vitro* sterile incubation of blood takes place also in blood sequestered for some time in the spleen. The data obtained in this experiment are presented in Figure 8 and Table III. It can be seen that there was a marked hemocon-

centration of the venous blood of the spleen as compared with the arterial blood. This is in accord with the findings of previous workers (29, 30). The EP was significantly higher in the venous blood from the spleen. As may be seen in Figure 8, the difference between the splenic venous and arterial EP content was always significantly greater than the difference between duplicate samples of the same blood. On the other hand, the EP content of the jugular vein blood taken prior to the administration of nembutal was the same as that of arterial blood after nembutal. It may be seen in Table IV that, except in dog No. 2, the values for ESFe in red cells from arterial blood were higher than in those from venous blood.

DISCUSSION

In these studies it was found that the free protoporphyrin of the erythrocytes (EP) rose simul-

TABLE IV
Effect of splenic stasis

	Hb art.	Hb ven.	Ht art.	Ht ven.	Retics. art.	Retics. ven.	†EP# jug. vein	†EP* art.	†EP* ven.	ESFe art.	ESFe ven.
	gm.	gm.	ml.	ml.	%	%	µg./100 ml. packed cells	µg./100 ml. packed cells	µg./100 ml. packed cells	% of Hb iron	% of Hb iron
Dog I	17.20	24.18	50.5	74.2	2.3	3.0		40 43	63.5 63.5	5.35 5.35	5.38 5.17
Dog II	14.63	21.00	42.0	60.3	—	—	17	16 16	25 27	5.16	5.26
Dog III	13.93	19.79	43.5	59.0	0.8	1.2		24.9 25.3	31.6 33.0	5.73	5.36
Pig I	16.50	22.25	49.0	63.0	1.3	2.0	127	134 134	138.0 137.5	3.06 3.20	2.99 3.02
Pig II	15.37	22.23	45.0	66.0	0.5	2.5	107	111 107	152 156	2.98 2.98	2.73 2.68
Pig III	13.76	19.92	40.1	61.0	3.3	4.9	133	126 129	260 258	2.87 3.04	2.62 2.63

†EP# The free erythrocyte protoporphyrin of the blood from the jugular vein taken before the administration of nembutal.

†EP* The free erythrocyte protoporphyrin of the arterial and venous blood after nembutal anesthesia. The determinations were made in duplicate and are so recorded.

taneously with or just prior to the increase in reticulocytes which developed following the administration of phenylhydrazine to rabbits, hemolysin to sheep, pyridoxine to pyridoxine-deficient pigs or folic acid to a patient with pernicious anemia in relapse. In no instance did an increase in EP occur following the rise in reticulocytes. When the content of free erythrocyte protoporphyrin (P_R , MRP) in the reticulocytes was compared with that in mature cells (P_E , MEP) the values were found to be strikingly and consistently higher in the immature forms. When the EP of red cells coming from the spleen following stasis was measured, it was found to be greater than that of cells from arterial blood. However, in the experimental anemias the EP did not rise when blood destruction was very great, increasing instead, as above noted, when young cells made their appearance.

These findings are consistent with those of Watson and his associates (4, 6), DeLangen and Grotepass (7) and Seggel (5), already cited, as well as with those of Stasney (31) who found the protoporphyrin content of the bone marrow to be increased when the percentage of normoblasts was increased. They support the suggestion that the free erythrocyte protoporphyrin represents porphyrin which is awaiting utilization for

hemoglobin formation. It is of interest in this connection that, not only was the mean reticulocyte content of protoporphyrin (MRP) higher than that of mature red corpuscles (MEP), but in a number of experiments the content in the reticulocytes which appeared at first was greater than in those appearing later (see Figures 1, 3 and 5). It might be expected that, in the reticulocytes appearing early in response to some stimulus, hemoglobin synthesis would not be as complete as in those which appeared when the crisis had become less acute.

It does not follow, however, that the presence of a high EP necessarily signifies the presence of a great number of immature cells. It would seem more correct to say, in the present state of our knowledge, that a high EP is indicative of uncompleted hemoglobin synthesis. The high EP found in iron-deficiency anemia (5, 8) may represent protoporphyrin which has not been formed into hemoglobin due to lack of iron; the high values observed in lead (4, 5, 32) and in gold (4) intoxication may represent a failure in hemoglobin synthesis due to chemical interference by these heavy metals. Finally the high EP observed in our laboratory (1) in cases of anemia associated with infection may be explained by assuming that there is a failure to utilize protoporphyrin completely for

hemoglobin synthesis. That there is failure in such cases of anemia to use iron in the normal fashion for this purpose has been shown by us already (33).

It seems possible also that under certain circumstances the free protoporphyrin in the erythrocytes may rise as the result of intracorporeal degradation of hemoglobin. The increase of EP following stasis of red corpuscles in the spleen, which we have observed, is similar to the increase noted by Watson *et al.* (4) *in vitro* and could be attributed to red cell degradation. Such an increase in EP could also be explained, however, by assuming that following splenic stasis a greater proportion of younger cells, containing more EP, remains, the oldest forms having been destroyed in the spleen. This thesis is consistent with the observation that the ESFe was lower in the venous blood than in arterial, but is not supported by the finding of a significantly increased percentage of reticulocytes in the blood from the splenic vein. Yet it is noteworthy that the hemolytic anemias produced in our experiments were not accompanied by a rise in EP at the time when red cell destruction was greatest.

We are at a loss to explain the discrepancy between our observations in pernicious anemia and those of Seggel (5) and of Watson *et al.* (4) who found the maximal increase in EP to follow rather than to coincide with or precede the maximal increase in reticulocytes. In phenylhydrazine anemia Watson *et al.* (4) observed a parallel increase in EP and reticulocytes. An increase in EP one day prior to the rise in reticulocytes, as we have observed in some instances, might have been missed by Watson *et al.* since their measurements were made at intervals of two or three days instead of daily. Speculation is unwarranted, however, until further studies are made to clarify this point.

With regard to the significance of the "easily split-off" iron (ESFe), it should be pointed out that (1) the ESFe increased within 24 hours following the injection of phenylhydrazine; (2) this increase coincided in one instance (Figure 5) with a rise in plasma iron, erythrocyte fragility and plasma bilirubin following the injection of hemolysin in one sheep but in another animal no increase was observed; (3) ESFe decreased at the time of maximal increase of reticulocytes; and

(4) the quantity in mature corpuscles (ESFe_E) was greater than in reticulocytes (ESFe_R). These observations suggest that ESFe is a degradation product of hemoglobin associated with destruction, maturation and perhaps senescence of red corpuscles.

The variations in plasma iron observed in our studies correspond to those described by others (34). An increase in plasma iron may result from its release from hemoglobin during blood destruction; rapid blood formation, however, leads to an increased demand for iron which in turn tends to deplete the amount in the plasma.

SUMMARY

1. The free protoporphyrin of the erythrocytes (EP) as well as the "easily split-off" iron (ESFe) were measured in reticulocytes and in mature cells taken from the blood of (1) rabbits in which anemia was produced by giving phenylhydrazine; (2) sheep in which anemia was caused by injecting anti-sheep red cell hemolysin; (3) pigs in which pyridoxine deficiency anemia was produced and subsequently treated; (4) dogs and pigs in which splenic stasis was induced; and (5) a human subject with pernicious anemia treated with pteroylglutamic acid.

2. It was found that the EP increased and reached a maximal level simultaneously with or just prior to the reticulocytosis observed in these conditions. The EP did not increase in the hemolytic anemias when the blood destruction was maximal. The content of EP was very much greater in reticulocytes than in mature red corpuscles. An increase in EP was also observed in the red cells taken from the splenic vein following stasis.

3. The ESFe increased in the first 24 hours following the injection of phenylhydrazine and then decreased at the time of maximal increase of reticulocytes. The content of ESFe in reticulocytes was less than in mature corpuscles.

4. These observations are interpreted as indicating that (a) an increase in EP usually signifies uncompleted hemoglobin synthesis which may be the consequence of the liberation of immature cells or is due to iron deficiency, toxic factors or other causes. An increase in EP may possibly also result from hemoglobin degradation; and

(b) the ESFe is a degradation product of hemoglobin associated with destruction, maturation and perhaps senescence of red corpuscles.

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THE SERUM CHOLESTEROL LEVEL OF THE PREMATURELY BORN INFANT AND ITS MOTHER

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Although Virchow (1) in 1847 had shown that the milky material in the blood of pregnant women was fatty in nature, it was not until 1911, that Neumann and Hermann (2) first actually demonstrated an increased total blood cholesterol during pregnancy. Both free and ester cholesterol were found to be increased in plasma and serum by Slemmons and Stander (3) and others (4 to 7). There is general agreement that the total cholesterol begins to rise early in the second trimester gradually reaching its peak at the eighth month, the average increase being approximately 25 per cent above normal.

In contrast to the high values found in the maternal circulation, it had been noted by Boyd and Wilson (8), Needham (9), Mayer (10), Schlossmann (11), and Muhlbock and Kaufmann (12), that the total cholesterol content of the fetal serum (or blood) was quite low. All except the first of these authors, therefore, have doubted the permeability of the human placenta to cholesterol.

Inasmuch as recent evidence suggests that cholesterol may be vital in steroid metabolism (13 to 16), it was decided in connection with related problems to investigate the cholesterol content of the serum of the premature infant and its mother. The author is not aware of any data in the literature on human subjects.

METHOD OF STUDY

All of the blood samples used in this study were obtained from patients in the delivery room at Parkland Hospital. There was no selection of cases, those reported being taken at random. Inasmuch as most of the patients entered the hospital in active labor, it was impossible to regulate the previous diet of the patient in order to control the length of time between the last meal and delivery.²

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² Review of the literature by Weinhouse (27) in 1943 on the influence of diet on cholesterol levels indicates that alimentary hypercholesterolemia after either ordinary

All newborns used in this study weighed under 2,500 grams. As soon as delivery was effected the umbilical cord was clamped and cut instead of waiting for the cord to stop pulsating in the orthodox manner. Fifteen cc. of blood were collected in a sterile tube from the placental end of the cord and allowed to clot. At the same time, 20 cc. of blood were drawn from the antecubital vein of the mother. Five cc. were reserved for a determination of the hematocrit reading, employing an isotonic anticoagulant. The remainder was allowed to clot and the serum after separation was refrigerated up to the time of the chemical analyses, which were in all cases started within 8 hours after delivery. Complete blood counts were made on the mother at the time of delivery.

The method used for determination of free and esterified cholesterol was that of Schoenheimer and Sperry (17) as modified by Sperry (18). It consists in principle of the precipitation of cholesterol with digitonin before and after saponification, after which the Liebermann-Burchard color reaction is used for the colorimetric determination, using a modified Duboscq colorimeter.

RESULTS

Table I shows the results obtained in the nine cases that have been studied. The total cholesterol of these prematures ranged from 51 mgs. per cent to 96 mgs. per cent with a mean of 71 mgs. per cent; whereas the mothers' values were 186 mgs. per cent to 383 mgs. per cent with a mean of 235 mgs. per cent. The free and combined fractions of cholesterol of the premature were rather constant, ranging from 25 per cent to 36 per cent, and 64 per cent to 75 per cent, respectively. The corresponding fractions for the mothers were 24 per cent to 39 per cent, and 61 per cent to 76 per cent. The averages of the partition for both mothers and immature infants are essentially identical. The lowest total cholesterol content (51 mgs. per cent) of the series was found in a baby weighing 2,268 grams, while the highest (96 mgs. per cent) was recorded in a newborn of only 1,270 grams.

meals or even meals containing considerable cholesterol has not been definitely proved.

TABLE I
Serum cholesterol content of premature infants and mothers in nine cases

	Serum cholesterol									
	Fetal cord blood						Mothers' blood			
		Sex	Total	Combined	Free	Combined	Total	Combined	Free	Combined
	<i>gms.</i>		<i>mgs. %</i>	<i>mgs. %</i>	<i>mgs. %</i>	<i>%</i>	<i>mgs. %</i>	<i>mgs. %</i>	<i>mgs. %</i>	<i>%</i>
1.	970	F	66.7	49.9	16.8	73	383	294	89	76
2.	1010	M	69.0	48.0	21.0	70	202	130	72	64
3.	1114	F	59.0	39.0	20.0	66	No blood collected	No blood collected	No blood collected	No blood collected
4.	1270	M	95.5	67.6	27.9	70	287	204	83	71
5.	1277	F	67.2	48.1	19.1	71	204	147	57	72
6.	1450	F	63.1	41.9	22.0	67	192	128	64	63
7.	2156	F	90.1	67.6	22.5	75	186	130	56	69
8.	2268	F	51.2	32.4	18.8	64	197	141	56	72
9.	2381	F	73.4	50.9	22.5	69	228	139	89	61
Average			70.6	50.2	21.2	69.3	235.1	164.1	70.7	69.7

DISCUSSION

It is noted that the relationship of free and esterified serum cholesterol to that of the total is remarkably constant, which confirms the findings obtained by Burger (19) Muhlbock and Kaufmann (12), Offenkrantz (20), and Smith and Marble (21).

It may also be noted that there is little, if any, change in cholesterol partition or absolute levels depending on the sex of the newborn. In addition it is seen that the serum cholesterol level of the immature infant is independent of its degree of prematurity and that it is for all purposes the same as that of a full term infant.

It appears from these data that during the last 2½ months of fetal life there is a low serum cholesterol and that the serum cholesterol of the mother bears no relationship to that of the fetus, so that permeability of the placenta to maternal cholesterol must be seriously questioned.

It has been noted by Gage (22), Mendel (23), and Baumann (24) that fat stained with Sudan III is stored when fed to pregnant animals. The fetus, however, shows no red color in its fatty tissue, thereby casting doubt on the transmission of fat across the membrane. In contrast to this work Boyd and Wilson (8) have shown very small arteriovenous differences in the lipid content of cord blood which they believe are due to transmission of small quantities of lipids to the fetus.

There has been no attempt made by any worker to explain the low cholesterol values found in the

newborn. It might be pointed out that after birth during the first 4 days of life there is a marked increase in total cholesterol, the values ranging from 90 to 140 mgs. per cent. After this the value remains fairly constant. Sperry (25) found the average value of total cholesterol from the fourth day on to be 133 mgs. per cent. This rise in cholesterol is converse to the estrogenic titer in the newborn which falls very rapidly immediately after birth, its only source having been the maternal blood stream. It has been reported by both Burger (19) and Muhlbock and Kaufmann (12) that there is a rise in the cholesterol content of the maternal blood during the puerperium, and that levels may exceed those reached during the eighth month. It has been noted by Levin (26) that large doses of stilbesterol markedly depress the cholesterol content of the blood plasma as well as that of the adrenals.

It was the author's observation (unpublished data) that the adrenals of the premature newborn have a markedly lowered cholesterol content. It may therefore be suggested that perhaps this lowered blood cholesterol of the newborn may be indirectly connected with the action of the estrogenic hormones. Further investigation of the validity of this hypothesis is in progress.

SUMMARY

1. The serum cholesterol values of the immature infant are the same as those of the full-term infants.

2. The absolute levels and partition of serum cholesterol do not vary with degree of prematurity.

3. The cholesterol levels of mother and infant bear no relationship to each other.

4. The free and ester fraction of cholesterol are found to be approximately 30 per cent and 70 per cent, respectively, in both the fetal and maternal serum.

5. It is suggested that the low cholesterol value obtained in the serum of the prematurely born infant and term newborn may be due to the depressant action of estrogenic hormones.

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DEPHOSPHORYLATION OF ADENOSINETRIPHOSPHATE BY NORMAL AND PATHOLOGICAL HUMAN SERA¹

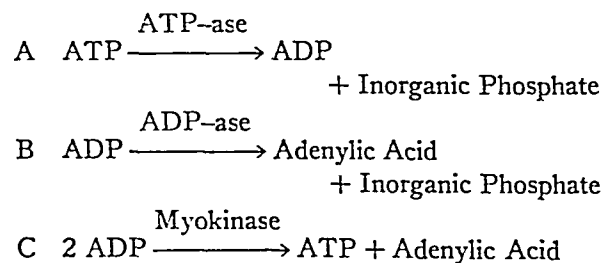
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INTRODUCTION

Recent studies indicate the importance of adenosinetriphosphate (ATP) in cellular metabolism. Adenosinetriphosphatase (ATP-ase), which catalyzes reaction A, has been found in a number of tissues.



Adenosinediphosphate (ADP) may be dephosphorylated to adenylic acid by adenosinediphosphatase (Reaction B). However, evidence for a specific ADP-ase is lacking and it is probable that the two-step dephosphorylation of ATP is catalyzed by the same enzyme (1). In muscle, ADP is converted to adenylic acid by the action of myokinase (1), in which labile phosphate is transferred from one molecule of ADP to another yielding one molecule each of ATP and adenylic acid (Reaction C).

ATP-ase has been found in muscle, liver, kidney, brain, other animal tissues, and in tumors (2), the highest concentrations being present in cardiac and skeletal muscle (3). Optimum ATP-ase activity has been found to occur at about pH 9 (3 to 7). In the course of an investigation of the dephosphorylation of ATP by human serum, a rather definite pH optimum was observed in acid media, as well as the expected optimum at alkaline reaction (8). It appeared that splitting of ATP at acid pH was not due to acid phosphomonoesterase. The dephosphorylation of ATP at acid and alkaline values of pH has been investigated in a small group of human sera.

¹ This study was carried out at Gallinger Municipal Hospital, Washington, D. C.

MATERIALS AND METHODS

Phosphorus was determined by the method of Fiske and Subbarow (9). Nitrogen analyses were carried out as described by Koch and McMeekin (10). Serum magnesium was determined according to a modification of the method of Denis (11). The glass electrode was employed for the measurement of pH.

ATP was prepared from rabbit muscle as the barium salt by the method of Kerr (12). This was converted to the sodium salt by precipitation with the stoichiometric amount of sulfuric acid, the solution then being adjusted to pH 7 with sodium hydroxide. The atomic ratio of nitrogen to phosphorus was 5.07:3, and that of acid-labile² to total phosphorus was 2.01:3. The muscle adenylic acid used in these experiments was prepared and characterized according to Ostern *et al.* (13), and further identified by the rate of color development with orcinol reagent (14).

Serum from patients and apparently healthy individuals was prepared from venous blood. Care was exercised to avoid hemolysis, which was found to increase the activity. In the studies reported, patients are referred to by number and the corresponding diagnoses are listed in Table I. The letter N appears before the numbers assigned to apparently healthy subjects.

Dephosphorylation of ATP was measured in a system designed to make the concentration of enzyme the rate-determining factor. The usual procedure was as follows: All solutions were brought to 37° before use. Five-tenths of a cubic centimeter of serum was added to 2.5 cc. of ATP in 0.1 M veronal-HCl or acetate buffer, and mixed immediately. At the end of a given period of incubation at 37° (usually from 0.5 to 2 hours), the reaction was stopped by the addition of 5 cc. of 8% trichloroacetic acid. The mixture was filtered and a suitable aliquot of the clear filtrate was analyzed for inorganic phosphorus and, in certain experiments, for acid-labile phosphorus. Controls with buffer and ATP alone, and with serum and buffer alone were employed. Determinations were usually carried out in duplicate. In some cases different concentrations of serum or incubation periods were employed in order to check the method. Where the effects of various salts were studied, these compounds were dissolved in the buffer-substrate solution, and controls containing these salts were used. Measurements of pH were carried out on identical mixtures at 37°. The data presented below are corrected

² Determined by hydrolysis with N HCl at 100° for 15 minutes.

Table 1
Decephalylation of ATP by Pathological Sera *

Case No.	Sex	Age	Diagnosis	Remarks	APP-ase pH 8.9 pH 4.8	Phosphatase Alkaline Acid
1	M	67	Carcinoma of prostate (b)	No metastases	0.56	0.51
2	M	72	Carcinoma of prostate (b)	Bony metastases; arthralgia	0.91	0.35
3	M	81	Carcinoma of prostate (b)	Bony metastases; arthralgia	0.43	0.32
4	M	73	Carcinoma of prostate (b)	No metastases; arthralgia	0.19	0.55
5	M	55	Carcinoma of prostate (b)	Bony metastases; arthralgia	1.84	0.67
6	M	76	Carcinoma of prostate (b)	Bony metastases; arthralgia	2.84	2.98
7	M	72	Carcinoma of prostate (b)	Bony metastases; arthralgia	3.02	1.66
8	M	61	Carcinoma of prostate (b)	Bony metastases; arthralgia	0.31	0.38
9	M	61	Carcinoma of prostate (a)	Metastases to bone and liver; arthralgia	7.84	2.84
10	M	73	Carcinoma of prostate (b)	Bony metastases; arthralgia	1.00	0.76
11	M	65	Carcinoma of prostate (b)	Bony metastases; arthralgia	0.44	0.40
12	M	56	Benign prostatic hypertrophy (b)	Bony metastases; arthralgia	0.46	0.42
13	M	68	Benign prostatic hypertrophy (b)	Bony metastases; arthralgia	0.23	0.40
14	M	67	Benign prostatic hypertrophy (b)	Bony metastases; arthralgia	0.93	0.50
15	M	63	Benign prostatic hypertrophy (b)	Bony metastases; arthralgia	0.31	0.37
16	M	65	Benign prostatic hypertrophy (b)	Bony metastases; arthralgia	0.43	0.35
17	M	40	Acute prostatitis (c)		0.26	0.32
18	M	34	Chronic prostatitis (c)		0.96	0.40
19	M	62	Chronic prostatitis (c)		0.42	0.35
20	M	60	Carcinoma of stomach (a)	Liver metastases; jaundice	3.71	1.60
21	M	65	Carcinoma of stomach (c)	Liver metastases	0.67	0.23
22	F	65	Carcinoma of stomach (a)	Liver metastases; jaundice	2.10	0.65
23	F	72	Carcinoma of stomach (a)	Liver metastases; jaundice	2.73	0.74
24	M	42	Carcinoma of stomach (c)		0.42	1.25
25	F	45	Carcinoma of breast (b)	Generalized metastases	1.99	1.94
26	M	75	Carcinoma of bladder (b)	Local extension	1.05	0.40
27	M	68	Carcinoma of colon (a)	Liver metastases	2.18	0.89
28	M	42	Carcinoma of larynx (b)		0.45	0.47
29	M	53	Carcinoma of lung (b)	No metastases	0.63	1.04
30	M	26	Hopkins disease (b)	Generalized	0.80	0.62
31	M	55	Posterior malpigny	Anemia, liver damage	3.45	2.42
32	F	49	Cirrhosis (c)	Mild liver damage	0.51	0.69
33	M	46	Cirrhosis (c)	Severe liver damage; jaundice	4.82	1.79
34	F	28	Cirrhosis (c)	Moderate liver damage	0.86	1.61
35	F	39	Cirrhosis (c)	Moderate liver damage	1.09	0.94
36	F	55	Cirrhosis (a)	Severe liver damage; jaundice	0.50	0.83
37	M	45	Cirrhosis (c)	Mild liver damage	0.53	0.35
38	F	64	Cirrhosis (c)	Moderate liver damage	0.37	0.77
39	F	60	Cirrhosis (c)	Severe liver damage; jaundice	0.65	0.77
40	M	40	Cirrhosis (a)	Moderate liver damage	3.16	0.73
41	M	45	Cirrhosis (a)	Severe liver damage; jaundice	0.92	0.47
42	F	45	Cirrhosis (c)	Mild liver damage	0.78	0.78
43	M	37	Cirrhosis (c)	Mild liver damage	1.20	0.79
44	F	58	Cirrhosis (c)	Mild liver damage	0.76	0.76
45	M	37	Cirrhosis (c)	Mild liver damage	0.65	0.75
46	M	67	Homologous serum hepatitis (a)	Severe liver damage; jaundice	0.95	0.05
47	F	34	Acute infectious hepatitis (c)	Mild liver damage	0.76	12.5
48	M	42	Acute infectious hepatitis (c)	Mild liver damage	0.53	0.53
49	F	25	Acute infectious hepatitis (c)	Mild liver damage	0.50	0.68
50	M	67	Congestive heart failure (c)	Arteriosclerosis	0.50	0.30
51	M	72	Congestive heart failure (c)	Arteriosclerosis	0.31	0.46
52	M	58	Congestive heart failure (c)	Arteriosclerosis	0.65	0.35
53	M	42	Syphilitic heart failure (c)	Aortic aneurysm	0.43	0.35
54	M	65	Congestive heart failure (c)	Arteriosclerosis	0.59	0.80
55	M	50	Congestive heart failure (c)	Arteriosclerosis	0.55	0.84
56	M	53	Congestive heart failure (c)	Arteriosclerosis	0.23	0.58
57	M	59	Subacute bacterial endocarditis	Arteriosclerosis	0.61	0.33
58	F	14	Subacute rheumatic fever (c)		0.59	7.5
59	M	15	Acute rheumatic fever (c)		1.30	0.86
60	M	40	Acute rheumatic fever (c)		0.76	0.89
61	M	34	Lobar pneumonia (c)		0.40	0.25
62	F	43	Lobar pneumonia (c)		0.26	0.39
63	M	23	Brachycephalus (c)		0.23	0.23
64	M	73	Congestive heart failure (c)	Arteriosclerosis	0.26	0.24
65	M	65	Lung abscess (c)		0.56	0.55
66	M	46	Silico-tuberculosis (c)		1.01	6.6
67	F	17	Peritonitis abscess (b)		0.22	3.5
68	M	22	Tetanus (c)		0.64	0.30
69	M	71	Tertiary syphilis (b)	Gumma of leg	0.53	0.72
70	M	59	Tobacco dysplasia (c)		0.27	0.60
71	F	35	Diabetes mellitus (c)		0.69	0.56
72	F	55	Diabetes mellitus (c)		0.83	0.41
73	M	75	Diabetes mellitus (c)		0.44	0.69
74	F	76	Diabetes mellitus (c)		0.28	0.39
75	F	46	Diabetes mellitus (c)		0.19	2.9
76	F	52	Diabetes mellitus (c)		0.23	2.4
77	M	53	Diabetes mellitus (c)		0.21	5.4
78	M	33	Diabetes mellitus (c)		0.15	3.5
79	M	47	Diabetes mellitus (c)		0.24	4.3
80	M	38	Diabetes mellitus (c)		0.37	0.49
81	F	31	Thyroiditis (c)	Receiving propylthiouracil	0.66	0.69
82	F	26	Thyroiditis (c)		0.79	1.14
83	M	42	Chronic glomerulonephritis (c)		0.33	0.89
84	M	42	Chronic glomerulonephritis (c)		0.29	0.37
85	M	43	Fracture of humerus (c)		0.90	0.54
86	M	30	Fracture of tibia (c)		0.43	0.62
87	M	48	Cerebrovascular accident (c)		0.50	0.54
88	M	65	Cerebrovascular accident (c)	Advanced pulmonary fibrosis	0.46	0.39
89	F	17	Boeck's sarcoid (b)		0.53	0.53
90	F	56	Myositis (b)		0.41	0.66
91	M	52	Pyopic ulcer (c)		0.34	0.43
92	M	40	Thrombotic purpura (c)	Splenectomy	0.32	0.75
93	M	14	Sickle cell anemia (c)		0.64	0.83
94	M	42	Myelogenous leukemia (c)		0.51	0.43
95	M	26	Scleroderma, generalized (c)		0.43	0.77

*APP-ase expressed as micromoles P split per cc serum per hour.

Acid and alkaline phosphatases are expressed as Guitman and Bodansky units per 100 cc serum, respectively.

†Receiving stilbestrol

(a)Diagnosis confirmed by autopsy

(b)Diagnosis confirmed by biopsy or operation

(c)Diagnosis based on clinical procedures

for serum inorganic phosphorus and nonenzymatic hydrolysis, and are expressed as micromoles of phosphorus split from ATP per cc. serum per hour, unless otherwise stated.

Serum alkaline and acid phosphatases were determined by the procedures of Bodansky (15) and Gutman (16), respectively, and are expressed in the customary units.

RESULTS

pH-activity curves

The liberation of inorganic phosphorus from ATP by human serum was found to exhibit optima in the ranges of pH from 4.6 to 5.1 and 8.7 to 9.1. This was true with normal serum and serum with increased activity (Figures 1 and 2). In normal serum the two optima are of about the same order of magnitude, although frequently the activity at

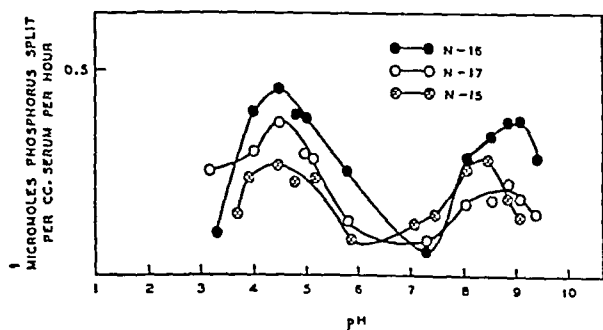


FIG. 1. pH-ACTIVITY CURVES FOR 3 NORMAL HUMAN SERA, CARRIED OUT WITH 9.67 MICROMOLES OF ATP

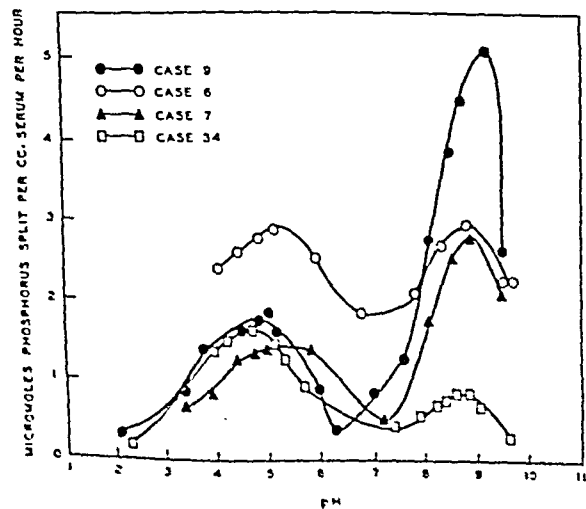


FIG. 2. pH-ACTIVITY CURVES FOR 4 PATHOLOGICAL HUMAN SERA WITH ELEVATED ACTIVITY, CARRIED OUT WITH 9.67 MICROMOLES OF ATP

acid pH was slightly greater. With certain pathological sera, however, the two activities were markedly different and showed no consistent relationship. The acid optimum was a constant finding in all human sera studied and similar data were obtained with 0.01 M citrate buffers and with three different ATP preparations. Identical pH-activity curves were observed with dialyzed serum. Subsequent studies were carried out at the pH optima, i.e., pH 4.8 and 8.9.

Effect of substrate concentration

Greater concentrations of substrate were found necessary for maximum activity at pH 4.8 than at 8.9 (Table II). Usually between two and

TABLE II

Effect of substrate concentration *

ATP conc. micromoles	Micromoles P split per cc. serum per hour	
	pH 4.8	pH 8.9
0.676	1.50	3.42
1.35	1.92	5.54
2.03	2.15	6.70
2.71	2.30	7.64
3.38	2.48	7.84
4.06	2.40	7.68
5.41	2.76	7.27
6.76	2.88	7.37
7.44	2.83	7.15
8.79	2.80	7.80
10.0	2.86	7.18
12.2	2.84	

* These data were obtained with serum of case 9.

three times as much ATP was required at pH 4.8 than at 8.9. In order to insure maximum activity, the studies described below were carried out with 3.38 and 9.67 micromoles of ATP at pH 8.9 and 4.8, respectively, except as noted in certain experiments. The liberation of inorganic phosphorus was directly proportional to serum concentration and duration of incubation. With sera of high activity, shorter incubation periods or lower serum concentrations were employed.

Dephosphorylation of ATP at pH 8.9

On prolonged incubation practically all of the ATP phosphorus was liberated as inorganic phosphorus at pH 8.9 (Figure 3, Table III). In addition the inorganic phosphorus produced was greater than the decrease in acid-labile phosphorus (Table III). The ratio of the decrease in

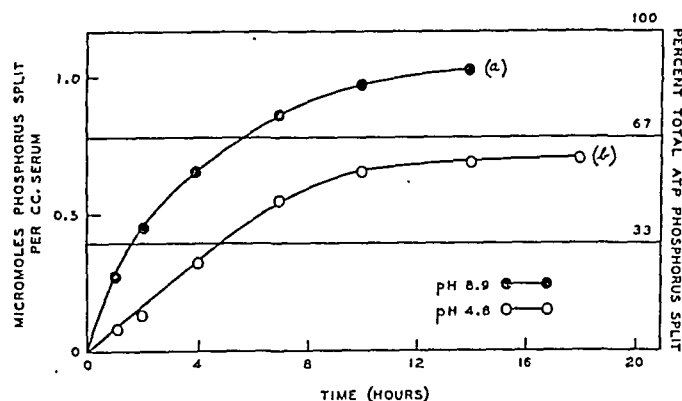


FIG. 3. DEPHOSPHORYLATION OF ATP BY NORMAL HUMAN SERUM (SUBJECT N-16) AT pH 8.9 (a) AND pH 4.8 (b)

Maximum hydrolysis is equivalent to 1.17 micromoles of inorganic P.

TABLE III
Dephosphorylation of ATP at pH 8.9 *

Time	Inorganic P (A)	Decrease in acid-labile P (B)	Ratio B/A	Per cent total P split
hours	micromoles	micromoles		
0.5	1.62	1.07	0.660	24.5
1.0	2.71	1.75	0.646	41.1
2.0	4.19	2.78	0.663	63.5
4.0	5.66	3.68	0.650	85.8
8.0	6.11	3.96	0.648	92.6

* These data were obtained with 1.0 cc. serum (case 5). Maximum hydrolysis \approx 6.60 micromoles inorganic P.

acid-labile phosphorus to inorganic phosphorus was, within experimental error, about two-thirds, suggesting that adenylic acid formed by splitting of labile phosphorus is completely hydrolyzed as soon as it appears. This was confirmed by studies in which serum was added to small amounts of adenylic acid under identical conditions of serum concentration, pH, and duration of incubation. The splitting of adenylic acid by normal and pathological sera was usually complete under these conditions (Table IV). The term "alkaline adenosinepolyphosphatase" (alkaline APP-ase) will be used to represent the enzymatic liberation of phosphorus from ATP at pH 8.9, since several enzymes, including phosphomonoesterase, may be concerned in the splitting of ATP.

Dephosphorylation of ATP at pH 4.8

The hydrolysis of ATP at pH 4.8 appeared to reach completion when about two-thirds of the total ATP phosphorus was split (Figure 3, curve

TABLE IV
Hydrolysis of adenylic acid *

Case No.	Alkaline phosphatase units	Acid phosphatase units	APP-ase		Adenylic acid		
			pH 8.9	pH 4.8	Added	Split	
						pH 8.9	pH 4.8
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
N-15			0.22	0.34	0.17	0.17	0.01
N-16			0.34	0.45	0.17	0.17	0
84	3.4	0.6	0.29	0.27	0.16	0.14	0
92	3.6	1.2	0.41	0.66	0.16	0.16	0.02
35	11.3	2.1	1.89	0.94	0.32 0.64	0.28 0.43	0.05 0.08
15	5.6	2.2	0.31	0.37	0.16	0.18	0
5	37.6	6.5	1.84	0.67	0.16 0.32 0.64	0.16 0.32 0.66	0.03 0.09 0.09
7	36.8	15.3	3.02	1.66	0.71	0.71	0.05
8	10.7	21.6	0.51	0.58	0.16 0.32	0.14 0.32	0.08 0.15
9	58.0	22.4	7.84	2.84	1.20 0.64	1.20	0.34 0.18
10	19.9	22.7	1.00	0.75	0.16 0.32	0.18 0.32	0.09 0.15

* Adenylic acid expressed as micromoles; experimental details given in text. APP-ase expressed as micromoles P split per cc. serum per hour.

b). The liberation of inorganic phosphorus was not appreciably greater than the decrease in acid-labile phosphorus (Table V), indicating that the splitting of ATP at pH 4.8 stops with the formation of adenylic acid. This is confirmed by the finding that negligible to small amounts of adenylic acid were split by most sera (Table IV). Greater, but not complete dephosphorylation of this sub-

TABLE V
Dephosphorylation of ATP at pH 4.8 *

Case No.	Time	Inorganic P	Decrease in acid-labile P
	hours	micromoles	micromoles
7	2	1.66	1.63
7	5	3.40	3.26
39	2	0.77	0.76
35	2	0.96	0.90
6	2	2.47	2.43
25	2	1.94	1.90

* These data were obtained with 0.5 cc. serum.

strate occurred with sera with elevated values of acid phosphatase associated with metastasizing carcinoma of the prostate. The enzymatic liberation of phosphorus from ATP at pH 4.8 will be designated as "acid adenosinepolyphosphatase" (acid APP-ase). In most cases this represents splitting of labile phosphorus and could therefore be termed adenylypyrophosphatase.

Effects of salts and dialysis

The effects of calcium chloride, magnesium sulfate, sodium fluoride, and sodium cyanide on serum APP-ase were studied (Table VI). No significant activation was observed in studies in which the concentration of calcium was varied between 0.1 and 0.0001 M. On the other hand, magnesium sulfate produced activation in some sera at pH 8.9, while there was no effect in acid media. Identical results were obtained with magnesium chloride. Activation by magnesium was quite variable and was usually observed in sera with elevated activity, but not in normal sera. When present,

TABLE VI
*Effect of salts **

Salt	Case No.	APP-ase pH 8.9		Case No.	APP-ase pH 4.8	
		Without salt	With salt		Without salt	With salt
CaCl ₂	N-5	0.31	0.36	N-15	0.29	0.29
	N-15	0.20	0.23	N-26	0.55	0.51
	6	1.73	1.80	9	2.48	2.49
	39	0.65	0.68	41	0.47	0.48
	5	2.01	2.05	42	0.78	0.71
	41	0.68	0.64	80	0.49	0.51
MgSO ₄	N-15	0.20	0.23	N-16	0.56	0.53
	N-22	0.26	0.26	N-5	0.32	0.28
	6	1.73	2.92	N-25	0.25	0.23
	39	0.65	1.14	7	1.78	1.70
	45	0.65	0.80	41	0.47	0.46
	7	2.76	3.58	9	2.40	2.36
NaF	22	2.02	2.16	22	0.65	0.62
	N-15	0.29	0.24	N-16	0.57	0.26
	N-5	0.29	0.27	N-5	0.32	0.16
	N-25	0.19	0.21	N-25	0.25	0.04
	33	4.82	4.65	24	1.17	0.51
	5	2.05	2.05	9	2.40	1.50
NaCN	45	0.65	0.64	22	0.65	0.25
	N-15	0.29	0.29	N-15	0.33	0.29
	N-5	0.29	0.28	N-5	0.32	0.32
	N-25	0.19	0.20	N-25	0.19	0.20
	41	0.76	0.61	24	1.17	1.17
	22	2.10	1.96	45	0.75	0.75
	9	4.18	3.48	9	2.10	2.10

* Concentration of added salt was 0.001 M. APP-ase expressed as micromoles P split per cc. serum per hour.

TABLE VII
*Effect of dialysis on serum APP-ase **

Case No.	pH 8.9		pH 4.8	
	Undialyzed	Dialyzed	Undialyzed	Dialyzed
N-25	0.19	0.23	0.27	0.28
N-17	0.22	0.21	0.50	0.49
21	0.66	0.67	0.23	0.26
42	0.78	0.60	0.78	0.82
41	0.92	0.99	0.54	0.59
40	3.16	3.17	0.73	0.70
9	5.17	5.17	1.77	1.89

* APP-ase expressed as micromoles P split per cc. serum per hour.

this effect was maximal with magnesium concentrations of 0.001 M. The reason for the variable effect of magnesium is not known. Determinations of serum magnesium revealed the reported normal levels of 1 to 3 mg. per 100 cc. (11), which would correspond to concentrations of 0.0000686 to 0.000206 M under the present conditions. Relatively high concentrations of magnesium (0.01 to 0.1 M) inhibited all sera studied. Sodium fluoride (0.001 M) produced marked inhibition at pH 4.8, while the same concentration of this salt had no appreciable effect at pH 8.9. Inhibition at pH 8.9 was noted, however, with 0.01 M fluoride. Sodium cyanide (0.001 M) inhibited sera of elevated activity but not normal sera at pH 8.9, while 0.01 M cyanide inhibited both normal and pathological sera. On the other hand, at pH 4.8, concentrations of 0.001 to 0.01 M cyanide had no significant effect.

In an attempt to determine the presence in serum of diffusible cofactors, activators, or inactivators, dialyzed sera were studied. Serum was dialyzed in cellophane sacs at 5° for 48 hours against large volumes of distilled water. The small precipitate which forms was resuspended. No appreciable difference in activity was found between the controls (refrigerated at 5°) and the dialyzed sera (Table VII). In addition no loss in activity occurred in sera stored for 48 hours at 5°, nor in sera frozen at -5° for three or four days.

Clinical studies

Studies of 27 apparently normal individuals are summarized in Table VIII. The group was about equally divided between the sexes and the range

TABLE VIII
APP-ase of normal human sera †

	pH 8.9	pH 4.8
Number of subjects	27	24
Range	0.13-0.66	0.20-0.66
Mean	0.31±0.1*	0.40±0.1

$$* \text{Std. dev.} = \sqrt{\frac{\sum d^2}{(n-1)}}$$

† APP-ase expressed as micromoles of $P_{\frac{1}{2}}$ split per cc. serum per hour.

of age was 20 to 63, the average age being 36. Significant variation with age or sex was not apparent, although a larger study would be necessary to investigate this point specifically. For the purposes of the present discussion, values greater than 0.70 at pH 8.9, and 0.80 at pH 4.8 are considered to be elevated.³

Of the pathological sera studied at pH 8.9, values above 0.70 were encountered in 31 cases, 23 of which exhibited clinical evidence of disease of liver or bone. All 13 cases with alkaline APP-ase greater than 1.30 had evidence of hepatic impairment or carcinoma of the prostate with bony metastases. Increased alkaline APP-ase was always associated with elevated alkaline phosphomonoesterase activity. All of the 31 cases with elevated alkaline APP-ase were found to have alkaline phosphatase values greater than 4 units, and 21 of these had values between 10 and 60 units. Although there were 29 cases with values of alkaline phosphatase greater than 4 units and normal levels of alkaline APP-ase, none of these exceeded 11 units and more than half were less than 6 units.

Of the pathological sera studied at pH 4.8, 19 were found to have APP-ase exceeding 0.80, and 12 of these (and all of the 8 cases with values greater than 1.50) had clinical evidence of hepatic or bone disease. It is now well established that marked elevations of acid phosphatase occur exclusively in cases of metastasizing carcinoma of the prostate, although slightly increased levels occasionally occur in other diseases (17, 18). Elevated acid phosphatase was present in 6 cases of prostatic carcinoma with bony metastases and in one case where no definite diagnosis could be made (case 31). These values are lower than

³ Values of acid and alkaline phosphatase, greater than 4 units per 100 cc., are considered to be elevated.

those of some of the reported cases probably because no patients with untreated advanced prostatic cancer were available for study. There appeared to be no consistent relationship between phosphomonoesterase and APP-ase at pH 4.8. Elevated acid APP-ase was encountered in cirrhosis and in non-prostatic cancer, where a normal level of acid phosphatase would be expected and was found. Of the 14 cases with elevated acid APP-ase, in which acid phosphatase was also determined, only four exhibited elevated acid phosphatase. In three cases of prostatic cancer with increased acid phosphatase, the acid APP-ase was within the normal range.

Slight variations in the data obtained on several normal individuals were noted over a period of 4 to 6 months. In patients with high values of APP-ase somewhat greater fluctuations with time were noted. Insufficient data are available, however, to relate these changes to the clinical course.

APP-ase of animal sera

The effect of hydrogen ion concentration on the serum APP-ase of three animal species was investigated. The sera of 4 dogs, 5 rabbits, and 4 specimens of pooled rat serum were studied. Representative pH-activity curves are given in Figure 4. Maximum activity occurred in the acid range with dog and rabbit sera, with only a small

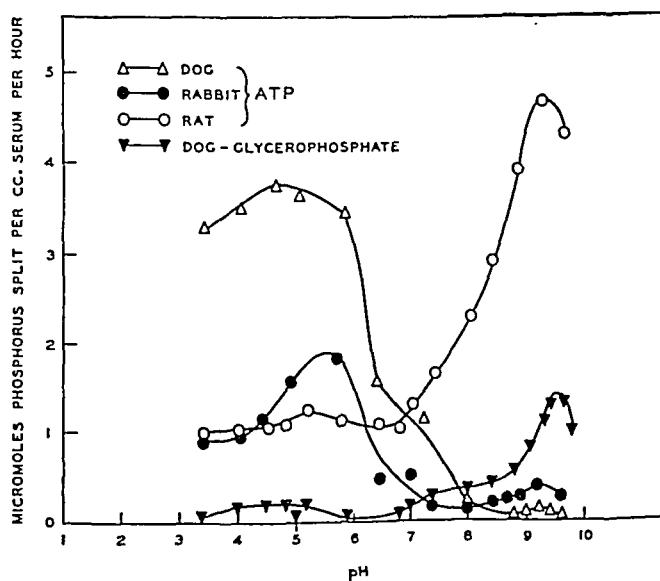


FIG. 4. pH-ACTIVITY CURVES FOR SERUM APP-ASE OF DOG, RABBIT, AND RAT, CARRIED OUT WITH 9.67 MICROMOLES OF ATP

The concentration of glycerophosphate was 0.025 M.

peak at about pH 9. In contrast, very high activity was found at pH 9 with rat serum and only a slight elevation in the rather flat curve in the acid region. As opposed to the results obtained for dog serum APP-ase, the pH-activity curve, with beta-glycerophosphate as the substrate, exhibited maximum activity at alkaline reaction. The latter pH-activity curve is very similar to those reported for normal human serum glycerophosphatase (19). Experiments in which mixtures of dog and human sera were tested for APP-ase activity at pH 4.8 yielded additive results.

DISCUSSION

Although a physical separation of acid and alkaline APP-ase was not attempted, it is probable that these activities represent different enzymes, as has been established for the phosphomonoesterases (20). There is no consistent relationship between the rates of dephosphorylation of ATP at pH 4.8 and 8.9 in various human and animal sera. More substrate is required to saturate acid APP-ase, and added salts (MgSO_4 , NaF, NaCN) have different effects on the two activities. The Russian workers have reported that in addition to the prominent pH optimum at 9, myosin preparations exhibit a second and lower optimum at pH 6.3, the entire pH-activity curve being equally lowered at all points by magnesium. A careful study led these authors to believe that the two optima were related to the dependence of activity upon the ionization of both enzyme and substrate, rather than to two different enzymes (21). This explanation does not appear applicable to the present results.

At pH 8.9, splitting of ATP could be catalyzed by ATP-ase, an ADP-ase or myokinase, and phosphomonoesterase. At this pH, adenylic acid is completely hydrolyzed and the rate-determining factor appears to be the splitting of labile phosphate. The values of adenylypyrophosphatase would therefore be two-thirds of those of APP-ase. Except with sera of increased acid phosphatase content, the values of acid APP-ase are equivalent to adenylypyrophosphatase activity.

The range of values of beta-glycerophosphatase activity reported for normal human serum (15, 19) corresponds to 0–0.35 and 0.48–1.28

micromoles of phosphorus per cc. serum per hour for acid and alkaline phosphatase, respectively. The corresponding normal serum APP-ase values are therefore somewhat less at pH 8.9 and slightly greater at pH 4.8 (Table VIII). With pathological sera, less phosphorus was also split from ATP than from beta-glycerophosphate at alkaline pH, and this difference was quite marked in certain cases. The acid APP-ase values, on the other hand, were higher than those of phenylphosphatase calculated on the same basis, in all but 3 (cases 5, 8, and 10) of the pathological sera studied.

The existence of a fairly specific ATP-ase active at alkaline pH is well established for muscle (6, 21). Purified myosin does not act upon inorganic pyrophosphate or monophosphates (6), but splits ATP, inosine triphosphate (22), and to some extent inorganic triphosphate (23). Jacobsen (24), Hasse (5), Barrenscheen and Lang (4), and Pillai (25) have reported evidence in support of a specific ATP-ase in liver, while Satoh (26) attributed the dephosphorylation of ATP to the combined action of pyrophosphatase and phosphomonoesterase.

At pH 8.9 the dephosphorylation of ATP could be due to several enzymes including alkaline phosphatase. However, in view of the lack of correlation between APP-ase and phosphomonoesterase at pH 4.8, it appears unlikely that acid phosphatase is responsible for ATP hydrolysis. Furthermore, with dog serum (Figure 4), the acid APP-ase was more than 16 times greater than the acid beta-glycerophosphatase activity. These findings are compatible with the recent work of MacLeod and Summerson (27). They report that a partially purified preparation of seminal fluid phosphatase was incapable of acting upon ATP, but they observed complete dephosphorylation of ATP by human seminal fluid. We have confirmed the latter finding, and have determined the pH optimum, which is in the neighborhood of 4, for the APP-ase activity of two specimens of seminal fluid.

An "acid" adenylypyrophosphatase, present in serum and seminal fluid, and possibly other tissues, is suggested by the present data. The question of specificity, however, remains to be investigated. Although acid phosphatase of prostatic origin does not act upon ATP, the possibility has not been ruled out that other phosphomonoesterases,

or even inorganic pyrophosphatase, may split ATP at acid pH.

The absence of activation by calcium is of interest in view of the striking calcium activation reported with myosin (6, 7, 21). Stimulation by calcium of ATP-ase but not beta-glycerophosphatase was reported by DuBois and Potter (3) for liver homogenates. Bailey (6) found that the activation by calcium of liver and electrical tissue ATP-ase was not as marked as with myosin. On the other hand, absence of calcium-activated ATP-ase was reported in rat muscle homogenates (28) and in chick embryos (29). It is possible that the concentration of calcium in serum is sufficient for maximum activity. Determinations of APP-ase of plasma prepared with 0.2% sodium oxalate revealed a slight and variable decrease in activity similar to the results reported for plasma phosphatase (15).

Magnesium increased the activity of certain pathological sera but had no effect with normal sera. Magnesium stimulates ATP hydrolysis by liver and electrical tissue (3, 6), and certain myosin preparations (6, 7), and has been shown to accelerate the conversion of ADP to adenylic acid (1, 6), and to activate alkaline phosphatase (30). Since adenylic acid, under our conditions, is completely split by serum at pH 8.9, in the absence of added magnesium, the most probable effect of magnesium is upon ADP breakdown. The inhibitory effect of higher concentrations of magnesium has also been observed in muscle and liver (3, 6).

Inactivation of ATP-ase by fluoride is well known. It is of interest that fluoride is a more efficient inhibitor at pH 4.8 than at 8.9, similar findings having been noted with acid phosphatase by Gutman (31). Cyanide has no effect on serum acid APP-ase but inhibits at alkaline pH. The latter finding was reported with 0.001 M sodium cyanide for liver ATP-ase (4), but not with chick embryo systems (32) or myosin (21, 33).

The phosphomonoesterases normally present in serum are believed to be derived from at least several body tissues. Additional serum alkaline phosphatase in certain diseases probably arises from the bones and liver, while malignant prostatic tissue is considered to be the source of marked elevations of serum acid phosphatase. In view of the fact that elevated serum APP-ase was associated mainly with liver damage and bony

metastases from prostatic carcinoma, it is quite possible that these tissues may be the source of the increased serum APP-ase. The variable effects of magnesium and cyanide with different sera may be due to qualitative differences in the APP-ase enzymes derived from different tissues, as has been noted for alkaline phosphatase (34, 35, 36).

In view of the striking species differences in serum APP-ase, studies of various human and animal tissues might be expected to exhibit similar variation. Although it is possible that determinations of serum APP-ase may be of some diagnostic value, it is apparent that a study of a larger number of patients, with the above mentioned and other diseases, is necessary for a more complete evaluation of these studies and their relationship to the usual clinical phosphatase determinations.

SUMMARY

The dephosphorylation of ATP by normal and pathological human sera exhibits two pH optima at about 8.9 and 4.8. Hydrolysis of ATP at pH 8.9 is associated with the splitting of all three phosphate bonds of ATP and is probably due to several enzymes including alkaline phosphomonoesterase, although the hydrolysis of labile phosphate is apparently the rate-determining factor. Dephosphorylation of ATP at pH 4.8 usually consists of splitting of the labile phosphate linkages, and the evidence suggests that this activity is not due to acid phosphatase. The existence of an "acid" adenylypyrophosphatase in serum, seminal fluid, and possibly other tissues, is suggested though not established.

The effects of substrate concentration, calcium, magnesium, fluoride, cyanide, and dialysis on the splitting of ATP by human serum are described. The pH-activity curves for the dephosphorylation of ATP by several animal sera are given.

A study of ATP dephosphorylation and phosphomonoesterase in a small group of normal and pathological human sera was made. Elevated adenosinepolyphosphatase activity was frequently associated with liver disease and bony metastasis from prostatic carcinoma.

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THE EFFECT OF EXERCISE ON THE RENAL PLASMA FLOW AND FILTRATION RATE OF NORMAL AND CARDIAC SUBJECTS¹

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In a previous communication (1) evidence was presented for a "forward failure" hypothesis of edema in patients who have low cardiac outputs at rest. These patients have a low renal blood flow, apparently accompanied by renal efferent arteriolar constriction. This seems to be directly related to the level of the cardiac output and is entirely unrelated to the height of the venous pressure. As the renal blood flow falls, a consequent reduction in filtration rate occurs. This results in a decrease in the amount of salt and water filtered, and, since the tubules continue to reabsorb salt and water almost completely, there is a net retention of the latter which produces edema. It is recognized that other factors may be involved in the rate of reabsorption. We emphasized that the patients studied were those who formed edema at rest, since the data were collected on resting subjects. Many patients who are compensated at rest form edema with activity. The reason for this can be ascertained only by studying patients in the exercising state. This paper is a report on the effect of exercise on the renal plasma flow and filtration rate of normal and cardiac subjects.

METHODS

Subjects were selected most of whom, at rest in the hospital, responded readily to routine therapeutic procedures. In this way it was hoped to obtain patients with relatively normal resting filtration rates. Controls consisted primarily of patients with asymptomatic neurosyphilis who were receiving penicillin therapy. Since other techniques are not suitable to demonstrate brief changes in renal plasma flow and filtration rate, the methods of Smith, Goldring and Chasis (2) were employed utilizing sodium para-amino hippurate for renal plasma flow and inulin for filtration rate.

As accurate results necessitate maintenance of a constant blood level of these materials, forms of exercise

were used in which a constant intravenous infusion could be given. At first, studies were made on recumbent patients with simple alternate flexion of each leg. As it became obvious that so little exercise was insufficient, the patients were required to step up and down two steps, each 12½ inches high, approximately 40 times. These patients were relatively free of edema as a result of mercurial diuresis before exercise was undertaken. Still later, in the recumbent position, pedals were pushed which, through two single pulley arrangements, raised two 22-pound weights alternately through a distance of 8 inches. Finally, as indicated in the table, 22-pound weights were raised through a distance of 12 inches. After allowing 30 minutes to acquire a constant blood level, a 12-minute exercise period was preceded by two 15-minute control periods and followed by sometimes one, usually two, 15-minute control periods. In most cases the normal subjects were required to do more work than the cardiac subjects. All results are corrected to a body surface area of 1.73 square meters.

In L. M., a patient with constrictive pericarditis, and M. T., a patient with heart failure associated with a chest deformity, who showed reductions in filtration rate with moderate exercise, the renal sodium excretion at absolute bed rest was compared with a day of walking about the ward, sweeping, etc. The same procedure was followed in another patient, S. L. H., with constrictive pericarditis, who failed to show a reduced filtration rate with the amount of exercise given.

The filtration fraction was calculated by division of the filtration rate figure by the renal plasma flow figure. An increase above 23 to 25 per cent indicates a rise in filtration pressure, best explained by efferent arteriolar constriction (3).

RESULTS

The results obtained in the present work should be considered in relation to results previously obtained in this program of study, wherein a correlation was attempted between the actual filtration rate and the tendency of patients to form edema. Filtration rates were determined on 42 ambulatory patients, all of whom had previously been in cardiac failure and were at the time on a regimen of digitalis, low salt diet and restricted activity. Twenty-eight of them required, in ad-

¹ Aided by grants from the Life Insurance Medical Research Fund and Smith, Kline and French Laboratories.

dition, the administration of mercurial diuretics once or twice a week in order to remain free of edema. The filtration rates in this group of cases are shown in Figure 1. Those who had to have diuretics are listed as "chronic," and those who did not, as "acute." It will be seen that all subjects with resting filtration rates below 80 cc. per minute required mercurial diuretics to keep them free of edema, whereas only three patients with filtration rates above 85 cc. per minute and none above 110 cc. per minute required mercurial diuretics. The amount of overlapping is surprisingly small in view of the considerable variation in activity and salt intake which undoubtedly existed. The critical level for salt retention under the conditions reported appears to be in the neighborhood of 70-80 cc. per minute.

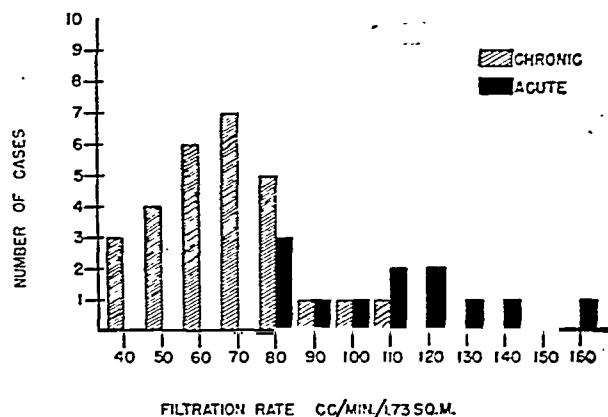


FIG. 1. FILTRATION RATES OF 42 AMBULATORY PATIENTS, PREVIOUSLY IN CARDIAC FAILURE

Those requiring diuretics listed as "chronic"; those who did not, "acute."

TABLE I

The effect of exercise on the renal plasma flow and filtration rate of normal and cardiac subjects.

Patient	Diagnosis	No. complete cycles	35l. per 1.73 sq. m. per min. (Each figure represents a 15-minute period)					
			Renal plasma flow			Filtration rate		
			Before exercise	During exercise	After exercise	Before exercise	During exercise	After exercise
22-lb. weight through 8 inches								
Controls								
E. J. L.	Convalescent pneumonia	100	615 482	421	405 425	125 113	114	99 106
F. W.	Meningovascular syphilis	160	530 574	570	570 568	100 103	104	104 99
Cardiacs								
L. V. E.	Syphilitic aortic insufficiency (rare diuretic required)*	183	250 232	239	239	87 94	98	93
O. P.	Syphilitic aortic insufficiency and arteriovenous aneurysm (diuretic 2 times weekly)*	160	270 260	57	233 257	73 73	16.4	74 84
22-lb. weight through 12 inches								
Controls								
R. S.	Asymptomatic neurosyphilis	150	483 490	438	423 415	128 115	103	109 110
A. G.	Asymptomatic neurosyphilis	200	367 375	336	297 298	128 127	141	130 142
G. D.	Asymptomatic neurosyphilis	295	536 483	570	475 500	122 111	127	103 113
Cardiacs								
L. B.	Hypertensive heart disease	135	215 209	51	193 197	94 88	25	106 97

TABLE I—Continued

Patient	Diagnosis	No.	Ml. per 1.73 sq. m. per min. (Each figure represents a 15-minute period)					
			Renal plasma flow			Filtration rate		
			Before exercise	During exercise	After exercise	Before exercise	During exercise	After exercise
Steps								
Controls								
F. G.	Asymptomatic neurosyphilis	80	568 518	495	539	126 123	126	127
N. D.	Asymptomatic neurosyphilis	84	408 393	264	318	119 102	91	96
D. M.	Asymptomatic neurosyphilis	88	759 755	442	820	149 152	103	182
E. C.	Asymptomatic neurosyphilis	82	405 415	306	322 350	126 117	96	111 114
R. R.	Asymptomatic neurosyphilis	88	434 448	384	452 448	103 113	107	114 106
H. D.	Asymptomatic neurosyphilis	88	724 663	436	404 760	167 161	115	103 172
Cardiacs								
M. T.	Heart failure due to chest deformity (diuretic 1 time weekly)*	62	406 384	204	448	135 131	76	159
L. M.	Constrictive pericarditis (diuretic 1 time weekly)*	80	521 370	138	412	133 95	37	114
S. L. H.	Constrictive pericarditis (no diuretic)*	84	654 676	527	502	139 147	135	129
L. V. E.	Syphilitic aortic insufficiency (rare diuretic)*	86	277 306	218	301 319	67 81	61	84 79
Horizontal leg movements								
Cardiacs								
M. B.	Hypertensive heart disease (diuretic 2 times weekly)*	100	123 98	66	131 135	69 61	31	63 60
W. F.	Rheumatic or syphilitic aortic insufficiency	100	241 233	171	307 246	85 86	58	102 86
W. R.	Syphilitic aortic insufficiency	100	280 233	258	242 258	75 73	82	79 83

* The frequency of mercurial diuretics refers to the patient in the ambulatory state. Variation in the amount of activity and variation in the salt content of the diet would probably modify this.

In reporting results with exercise, therefore, primary interest is centered on the question of whether or not the filtration rate fell below that point. In most cases the fall in filtration rate paralleled the decrease in renal plasma flow so that no comment on the latter is made in the text. Changes of less than 25 per cent are considered

within the limits of technical error of the procedure.

No controls were studied using simple leg flexion in the recumbent position and the controls employing the light weights may be used for comparison. The filtration rate of one cardiac subject (W. R.) with horizontal leg flexion remained

unchanged. That of another (W. F.) fell significantly but the subsequent control period level greatly exceeded the others and it is believed that the bladder may have been incompletely emptied during the exercise period, giving a false low value. A third had a resting average of 65 cc. per minute and fell to 31 cc. per minute with exercise.

Two control subjects were studied in the horizontal position with 22-pound weights moved through a distance of 8 inches. One of these showed no change and the other had a slight de-

crease in renal plasma flow and none in filtration rate. The renal studies of one cardiac subject were unaffected by the amount of exercise given while the other showed a striking 75 per cent reduction in plasma flow and filtration rate, the latter falling from 73 to 16 cc. per minute.

Six normal individuals were studied utilizing steps (Figure 2a and b). Each point in the figures represents the result of a 15-minute period. Two were unchanged but four had an appreciable drop in renal plasma flow during the exercise pe-

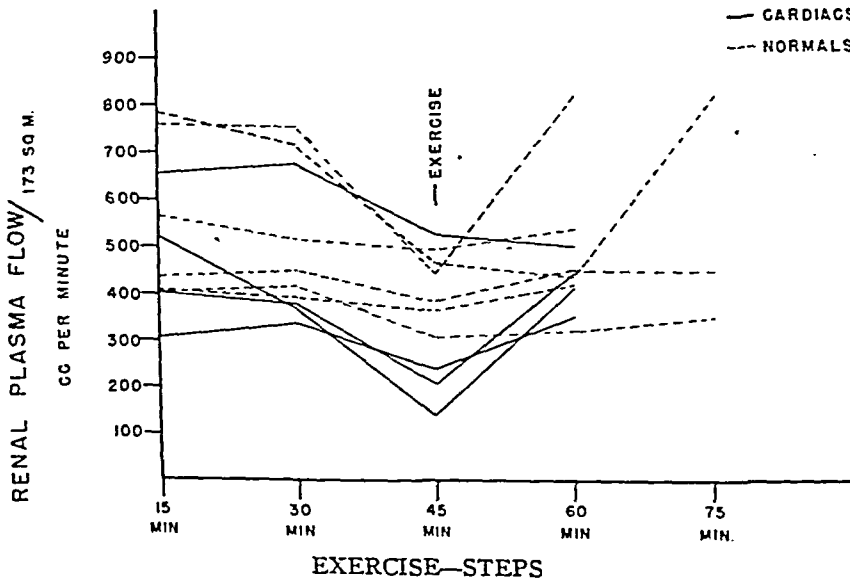


FIG. 2a. RENAL PLASMA FLOW OF 6 NORMALS AND 4 CARDIAC PATIENTS IN THE STEP EXERCISE

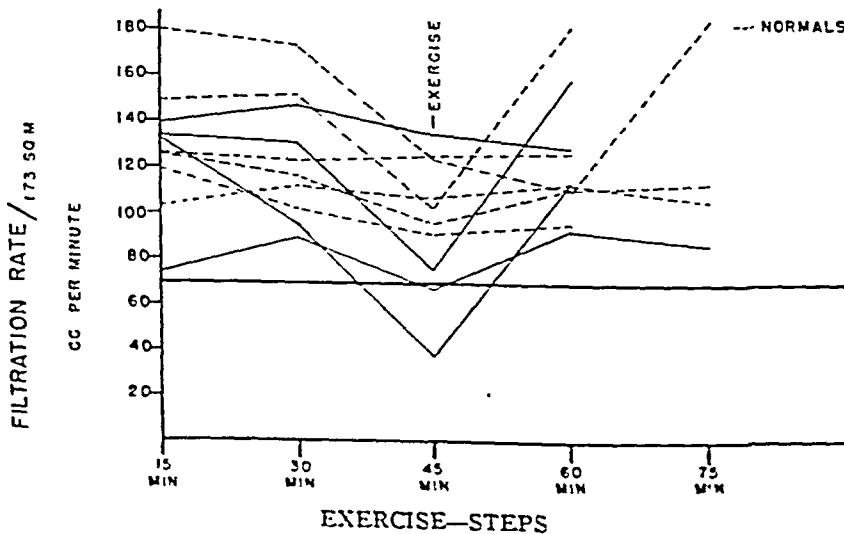


FIG. 2b. FILTRATION RATES FOR THE SAME

riod. The filtration rates also fell but none approached the critical level of 70 cc. per minute, the lowest being 91 cc. per minute. Of four patients with cardiac failure, one had no change in renal function with exercise, one had a slight decrease, one exhibited a 50 per cent drop, and one had a fall of 75 per cent. Of the last three, the first had a resting filtration rate of 74 and dropped to 61 cc. per minute. The filtration rate of the second fell from 134 to 76 cc. per minute, despite the fact that she did only 75 per cent of the required amount of exercise. The other had a marked fall from a mean of 119 to 37 cc. per minute.

Three normal individuals were able to push the 22-pound weights through a distance of 12 inches for 15 minutes without appreciable change in renal function. One did twice as much exercise as the cardiac patient. The cardiac subject had a striking decrease from 92 cc. to 25 cc. per minute.

Filtration fractions tended to rise with exercise. This was more evident in the control subjects, some of whom had rather marked drops in renal plasma flow with little change in filtration rate, suggesting efferent arteriolar constriction.

The two cardiac patients whose filtration rates fell with exercise had striking reductions in sodium output during 12 hours of walking about the ward, sweeping, etc., as compared to a 12-hour period of rest in bed. The sodium output of one fell from 104.0 mEq. to 57.4 mEq. and the other from 84.6 mEq. to 33.4 mEq. The cardiac subject who had no reduction in filtration rate with exercise had a rise in sodium output from 50.2 mEq. during a rest day to 73.4 mEq. during a day of activity. This patient had constrictive pericarditis with a venous pressure of 27 cm. of saline.

DISCUSSION

In interpreting the data presented here it must be remembered that various factors may cause an apparent absence of response of the renal blood flow to exercise, under the conditions of these methods. First, the limited amount of exercise which is possible under the experimental conditions may not tax the cardiac reserve sufficiently to produce a renal shutdown. If a patient has a normal resting renal plasma flow, as was the case with S. L. H., a fall of renal plasma flow in response to mild exercise would not be likely to oc-

cur. Marked exercise, such as running the 440-yard dash, will produce a renal shutdown even in normal individuals, as has been demonstrated by crude techniques (4). Another group of patients in which there might not be a response consists of those whose renal shutdown is already quite marked and in whom further mild stimulus might not produce a greater change. Such a case is L. V. E. However, this is not always true (see M. B.). A third group in which exercise might not be expected to lower the filtration rate is exemplified by patients with renal disease in whom salt and water retention is caused by either destruction of entire nephrons or interference with filtration by thickening of Bowman's capsule. Such a change occurs in glomerulonephritis. We have observed one such patient for a period of three years. The real test of the validity of the data is whether there is a significant reduction in the filtration rate toward the critical level for edema formation as compared to the response of the filtration rate of the normal controls. A response to light exercise in all cardiac patients selected will probably not be demonstrable until more sensitive methods of study can be applied to the selection of patients, or until we know how the renal shutdown is mediated from the stimulus of *inadequate* cardiac output. Such knowledge may bring recognition of other factors not mentioned here.

In general, patients with filtration rates below 70 cc. per minute who have normal tubular reabsorption of sodium tend to retain salt and water with an average salt intake (see "Results"). Most of the cardiac subjects in this study had resting filtration rates above 70 cc. per minute though many had a marked depression of renal plasma flow. Since the diminution in renal plasma flow and filtration rate has been shown to be related to the cardiac output (1) they must have had cardiac outputs adequate to prevent the formation of edema at rest. Even with the small amount of exercise performed in these experiments, however, many of the subjects were apparently unable to increase the cardiac output sufficiently to maintain a normal circulation in the face of the increased demands of the body. Such a situation seems to produce renal vasoconstriction (1).

The cause of the renal vasoconstriction accompanying an inadequate cardiac output is un-

known. It could be a sympathetic nervous stimulation from the tissues or central nervous system. Preliminary studies in sympathectomized individuals and a patient with orthostatic hypotension indicate that this is not true (5). One would not expect a primarily renin effect in individuals with normal or elevated cardiac outputs. It could be an adrenalin effect. Stimulation from some metabolite from the tissues or some humoral substance from a specialized tissue are possibilities. Work is in progress to clarify this problem.

SUMMARY

1. Patients with heart failure who form edema at rest usually have a low resting cardiac output and a correlatively low resting renal plasma flow with a filtration rate below 70–80 cc. per minute. Since tubular reabsorption is almost complete, the low filtration of salt and water results in retention of salt and water, *i.e.*, edema. The operation of other factors in sodium reabsorption is appreciated.

2. Cardiac subjects who form edema only while exercising usually have filtration rates above 70 cc. per minute. In order to determine why they form edema it was necessary to study them under the conditions in which the edema was formed—in the exercising state.

3. With various forms of mild exercise, the filtration rates of six of 10 cardiac subjects approached or fell well below the "critical" level of 70 cc. per minute. None of the control subjects showed a comparable change in filtration rate, though a few had a definite fall in renal plasma flow.

4. Thus there seems to be a mechanism for reducing the renal plasma flow when the cardiac output is insufficient for tissue demands, perhaps in order to supply tissues such as the brain, the metabolic needs of which are greater than those of the kidney in proportion to blood supply. The possible mechanisms of this are mentioned.

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SENSITIVITY OF THE TUBERCLE BACILLUS TO STREPTOMYCIN BEFORE AND DURING SPECIFIC THERAPY^{1, 2, 3}

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Shortly after the demonstration of a therapeutic effect of streptomycin upon tuberculous infections in animals (1, 2) and in the human (3), Youmans and his co-workers (4) found that cultures from patients subsequent to such specific therapy yielded organisms which were resistant to streptomycin. These investigators not only observed that 7 of 8 strains recovered from 12 patients before and after treatment with streptomycin exhibited an increase in resistance of from 500- to 1,000-fold compared to tests performed prior to initiation of therapy, but they also showed that the resistance of two strains of *Mycobacterium tuberculosis*, one of the human type and one of the avian type, increased more than 1,000-fold when the organisms were exposed to streptomycin *in vitro*.

The development of such resistance, or perhaps better expressed as loss of sensitivity, of the tubercle bacillus during streptomycin therapy has recently been studied in a joint program of the Yale University School of Medicine and the Laurel Heights Sanatorium, carried out under the auspices of the American Trudeau Society. The results of this study are presented below.

¹ Presented in part at a symposium on streptomycin therapy of pulmonary tuberculosis before the Yale Medical Society, New Haven, May 14, 1947; and at the Eastern Sectional Meeting of the American Federation for Clinical Research, New York City, December 12, 1947.

² This study was aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

³ This study is part of the Streptomycin-Tuberculosis Research Project of the American Trudeau Society, Medical Section, National Tuberculosis Association. The drug was generously donated to the Society by the following: Abbott Laboratories, Eli Lilly & Co., Merck & Co., Inc., Charles Pfizer & Co., Inc., E. R. Squibb & Sons, and the Upjohn Co. Collateral clinical observations were made at the Laurel Heights Sanatorium by Dr. Kirby S. Howlett, Jr., and Dr. John B. O'Connor to whom the authors are indebted for generous advice and assistance with the reported study.

METHODS

A group of 16 patients with pulmonary tuberculosis was treated with 1.8 grams per day of streptomycin in 6 divided doses until late in the course of treatment when the same daily amount of streptomycin was divided into 5 doses, omitting the 3:00 a.m. dose. In most instances, treatment was discontinued after four months. Eight of these patients also had ulcerative tuberculous bronchitis.

Sputa or gastric washings were collected at routine intervals. Tubercle bacilli were isolated from these specimens by inoculation of the neutralized alkaline concentrate on Hohn's and egg yolk media. After satisfactory growth had been obtained, transfer of part of a suitable colony was made to a tube of modified Dubos-Davis medium (5, 6) with a nichrome spatula, mashing and triturating the colony in the medium in order to give as diffuse a suspension as possible. Later on in the work it was found desirable routinely to add penicillin (20 units per cc.) to the Dubos medium used for first transfer from the solid slants in order to prevent contamination with gram positive cocci. After a growth period of from 7 to 10 days at 37° C., tubercle bacilli grew in a lacey pattern at the bottom of the tube in this initial transfer. In subsequent transfers, such growth appeared as early as the third or fourth day. When growth was sufficient after shaking to cause turbidity approximately equal to that of the No. 2 McFarland nephelometer scale, the culture tube was well shaken and allowed to stand for 15 minutes to allow larger particles to settle out. Pyrex tubes, each containing 5.0 cc. of the Dubos-Davis medium to which had been added the requisite quantity of streptomycin, were then inoculated with 0.1 cc. of the turbid supernatant culture. The following concentrations of streptomycin were routinely employed: 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0, and 1,000.0 mcg. per cc. In several instances, concentrations of 2.0, 3.0, and 4.0 mcg. per cc. were also used. Daily readings were made from the fourth to the 12th days, the end point being taken as the tube containing the lowest concentration of streptomycin which was sufficient to inhibit the growth of tubercle bacilli. Ordinarily, no change was observed after the 7th day of incubation. Growth of tubercle bacilli was confirmed by acid fast stain of the culture.

Pyrex tubes used in the experiment were 25 × 150 mm. in size. Precautions concerning chemical cleanliness of these tubes and the preparation of cotton plugs as outlined by Dubos and Davis (5) were carefully observed.

Details of the preparation of the modified Dubos-Davis medium are given below:

1. Mineral Concentrate

Sodium phosphate, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	— 12.5 grams
Potassium phosphate, KH_2PO_4	— 2.0 grams
Sodium citrate, $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$	— 3.0 grams
Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	— 1.2 grams
Distilled water	— 100.0 cc.

Adjust to pH 7.2

2. Stock Medium

Mineral concentrate (No. 1)	— 100.0 cc.
N-Z Amine, ⁴ 5% in dist. water	— 40.0 cc.
VegeX, ⁵ 5% in dist. water	— 4.0 cc.
Ferric ammonium citrate	— 0.10 gram
Asparagine	— 2.0 grams
Distilled water	— 800.0 cc.

Store in stoppered flask in ice box, adding about 5 cc. of chloroform as "puddle" in bottom of flask. This solution may be thus stored for several months.

3. Tween 80 (lot 1405),⁶ 10% in dist. water. Keep in ice box for not longer than 1 week before using.

4. Dextrose, 25% in dist. water, autoclaved at 15 lb. for 30 minutes.

5. Albumen⁷ 5%, bovine plasma fraction V in 0.85% sodium chloride. Neutralize to pH 7.0 with sodium hydroxide, filter through Seitz filter under aseptic conditions, heat to 55° C. for 30 minutes in water bath in order to inactivate lipase.

6. Final Medium

Add 0.2 cc. of 10% Tween 80 solution (No. 3) to 100 cc. of stock medium (No. 2). Autoclave at 15 lb. for 20 minutes; when cool, add 2.0 cc. of 25% dextrose solution (No. 4) and 10.0 cc. of 5% albumen solution (No. 5). Dispense 5.0 cc. each in pyrex tubes (25 × 150 mm.) with requisite concentrations of streptomycin.

RESULTS

Strains of tubercle bacilli recovered from the group of 16 patients suffering from pulmonary tuberculosis and treated with streptomycin were tested for their sensitivity to the drug in the manner described above. The results are presented in Table I.

All 16 strains of the tubercle bacillus recovered prior to treatment were highly susceptible to the *in vitro* action of streptomycin. Fourteen of these strains were inhibited by a concentration of

TABLE I

Streptomycin sensitivity of strains of tubercle bacilli isolated from 16 cases of pulmonary tuberculosis treated with 1.8 grams of streptomycin per day for four months. The open spaces indicate failure to isolate organisms by culture from the sputa or gastric washings

Patient	Pre-treatment mcg./cc.	End of 1st month mcg./cc.	End of 2nd month mcg./cc.	End of 3rd month mcg./cc.	End of 4th month mcg./cc.
Case 1—Eh	0.5		1.0		
Case 2—Ge	0.5	5.0	50.0	0.5	10.0
Case 3—Gi	0.5		10.0		
Case 4—Ko	0.5	5.0	3.0	5.0	5.0
Case 5—Ma	0.5	0.5	5.0	>1000.0	
Case 6—Mi	0.5	0.5	0.5	5.0	5.0
Case 7—Ob	0.5	0.5			
Case 8—Re	0.5	0.5			10.0
Case 9—Ro	0.5	0.5			
Case 10—Si	0.5	1.0	500.0	500.0	
Case 11—So	1.0	0.5	0.5		
Case 12—Su	0.5	0.5			
Case 13—Vel	0.5	0.5	0.5		
Case 14—Ver	1.0	0.5	0.5		50.0
Case 15—Wa	0.5	0.5		50.0	
Case 16—Wi	0.5	5.0	4.0	>1000.0	>1000.0

0.5 mcg. of streptomycin per cc.; the remaining two strains were inhibited by 1.0 mcg. of the agent per cc.

At the end of the first month of therapy, isolation of the organism by culture was successful in 14 of the cases. Except for three instances where there was a ten-fold increase in resistance to streptomycin, there was no significant change in reaction to the *in vitro* effect of streptomycin of the remaining strains.

By the end of the second month of therapy, not only did attempts at isolation of the tubercle bacilli from sputa or gastric contents reveal an increasing number of negative specimens, but also an increasing frequency and degree of resistance of the organisms to the chemotherapeutic agent. For instance, negative cultures had increased from 2 after the first month of therapy to 5 by the end of the second month. Along with this, 4 of the 11 isolated strains now showed a ten-fold or greater increase in resistance. In 2 of these 4 resistant strains, resistance had increased 100-fold and 1000-fold, respectively.

During the third and fourth months of therapy the number of negative specimens and resistance of those organisms obtained by culture continued to increase. At the end of the third month, positive cultures were obtained in 7 of the 16 cases. In all but one instance, a ten-fold or greater in-

⁴ Sheffield Farms Co., 524 West 57th Street, New York City.

⁵ Vegex Company, 175 Fifth Avenue, New York City.

⁶ "Tween 80" is the trade name of a polyoxyethylene derivative of sorbitan monooleate. It was furnished through the courtesy of the Atlas Powder Company, Wilmington, Delaware.

⁷ Armour Laboratories, Chicago, Illinois.

crease in resistance was demonstrated. Two of the 6 resistant strains showed over a 2,000-fold increase, while tests on the remaining 4 of these 6 strains indicated that resistance had increased ten-fold in two instances, and 100-fold and 1,000-fold in the remaining two, respectively.

By the end of the course of therapy at the fourth month, 6 positive cultures were obtained. Tests of these organisms showed a ten-fold increase in resistance in two cases, 20-fold in two cases, 50-fold in one case and over 2,000-fold in the remaining case.

DISCUSSION

The results described above show a general tendency toward a steady reduction in the number of culturally positive sputa and gastric contents while the patients were under treatment with streptomycin. They further demonstrate the progressive development of significant streptomycin resistance of remaining organisms during such specific therapy. It should be stated at this point that the term "development of resistance" is loosely used throughout this report; the authors do not imply that a given genetic strain actually "develops" resistance but merely that resistant organisms appear. A discussion of whether this "resistance" depends upon the selection of genetically resistant substrains in a culture, as has been shown with other micro-organisms and other chemotherapeutic agents, or whether true resistance of a given genetic strain of the *Mycobacterium tuberculosis* actually develops during exposure to streptomycin is beyond the scope of the present communication. Pyle (7) has very recently brought forth evidence which indicates that the former suggestion is correct.

It is of considerable interest that all organisms isolated after the third or fourth month of streptomycin therapy in the present study showed at least a ten-fold increase in resistance. This holds for 9 of the 16 cases investigated. In the remaining 7 cases, cultures had reverted to negative by this period.

Breakdown of the 9 strains of organisms isolated after the third or fourth month of therapy indicates the following degree of development of resistance when compared to the sensitivity of the strain isolated prior to treatment:

10-fold increase in resistance—2
20-fold increase in resistance—2
50-fold increase in resistance—1
100-fold increase in resistance—1
1,000-fold increase in resistance—1
2,000-fold increase in resistance—2

Total number of strains isolated at end of therapy —9

The clinical significance of this development of resistance is not clear at the present time. Indeed, the exact level at which organisms may be called "resistant" has not even been defined. While there is no conclusive clinical evidence in the human to indicate that *in vivo* and *in vitro* resistance parallel each other, Youmans and Williston (8) have shown that tuberculous infections produced in mice with streptomycin-resistant cultures of the tubercle bacillus were not suppressed by treatment of the animals with streptomycin.

Although it is not possible to attempt any definite correlation between the development of resistant organisms and the clinical course of the patients treated in the present study the following general statements, particularly with reference to the four cases in which resistance had increased at least 100-fold, may be made (9):

1. Definite relapse (clinical, radiologic, and bronchoscopic) occurred under treatment in one patient at about the time that the tested organisms showed a 2,000-fold increase in resistance.

2. Of the other three cases showing highly resistant strains during treatment, two continued to improve satisfactorily; the other case never showed significant improvement at any time.

3. One patient who relapsed under treatment (fever, increased expectoration, recurrence of grossly positive sputum, but no unfavorable x-ray change) and another patient who failed to maintain peak improvement, each yielded a strain of organism showing only a ten-fold increase in resistance.

It is evident from the foregoing data that it is impossible at this time to draw any definite correlation between *in vitro* resistance of the causative organism and the therapeutic course in the small number of cases treated. Further observations with respect to this correlation (9) and data concerning the resistance of organisms to strep-

streptomycin in the post-treatment period (10) will be recorded at a later date.

While investigators agree that tubercle bacilli develop a variable degree of resistance to streptomycin during therapy, they are not all in harmony as to the number which do so. Part of the disagreement, at least, may be due to two factors: first, definition of the term "resistance," and second, use of different technical procedures and media for the assay of resistance. Youmans *et al.* (4) first reported the development of resistance; they found that tubercle bacilli isolated from 8 of 12 patients after treatment with streptomycin showed a marked resistance to streptomycin. Resistance of 7 of these 8 strains, when compared with the resistance of strains isolated from the same patients before treatment, had increased 500- to 1,000-fold. These findings have been very recently confirmed by Youmans and Karlson (11). McDermott's observations (6) indicate that virtually all strains isolated after the 12th week of therapy gave sensitivity values of 100 mcg. or higher (equivalent to an increase in resistance of approximately 200-fold or higher). In some instances this resistance developed as early as the end of the first month of therapy. Studies carried out under the auspices of the Veterans Administration (12) show that 78% of positive cultures obtained from streptomycin-treated patients at the end of 120 days contained organisms which were resistant to more than 10 mcg. of streptomycin per cc. (20-fold increase).

In striking contrast to the above observations which are in accord with the findings in the presently reported paper, Wallace and Fisher (13) found that only 4 of 20 strains (20%), isolated after four months of streptomycin therapy, manifested resistance to the drug when tested in Dubos medium. Resistance was defined as growth in 10 mcg. or more streptomycin per cc. of medium. Utilizing Youman's medium (11) as the test medium for determination of sensitivity of these same tubercle bacilli to streptomycin, Wallace and Fisher now found that 11 of the 20 strains (55%) could be classed as resistant, using the definition as given above.

It is possible that the low incidence of appearance of streptomycin-resistant strains reported by Wallace and Fisher with Dubos medium may be due to the use of a medium with a relatively low

concentration of albumin and a relatively high concentration of Tween 80, thus giving falsely low values for the streptomycin sensitivity of tubercle bacilli. In our experiments, albumen concentrations of 0.5% and Tween 80 concentrations of 0.02% in the final medium were employed, thus duplicating the conditions advised by McDermott and his collaborators (6) who have particularly stressed the necessity of using a low concentration of Tween 80 since this agent appears to possess a bacteriostatic action upon the growth of the tubercle bacillus.

In view of the disagreement noted above, it is also natural to inquire as to the possibility of selection of colonies of varying degrees of resistance to streptomycin from the same culture, a phenomenon previously described with non-acid fast organisms. In a number of instances, attempts were made to check the sensitivities of some of the strains of tubercle bacilli described in Table I, utilizing the same culture but different colonies. In some instances cultures isolated within a week or two of the reported strains were also employed. The results show that while the values obtained were in general agreement (within limits of technical error) with the values previously obtained, there were notable exceptions not only to the specific sensitivity value for a given date but also to the general trend. This point obviously needs further clarification since the authors are satisfied with the relative accuracy of the procedure outlined for determination of sensitivity.

SUMMARY

In vitro streptomycin sensitivity of tubercle bacilli isolated from sputa or gastric contents was determined in a group of 16 patients with pulmonary tuberculosis receiving 1.8 grams of streptomycin daily for a period of four months. *In vitro* tests were performed in a modified Dubos-Davis medium.

In all 16 cases, the strains of bacilli isolated prior to treatment were highly sensitive to streptomycin. Fourteen of these 16 strains were inhibited by 0.5 mcg. of streptomycin per cc., the remaining 2 strains were inhibited by 1.0 mcg. per cc.

Loss of sensitivity (ten-fold increase in resistance) began to appear by the end of the first month of therapy together with conversion of

positive sputum or gastric washings to negative in other cases as determined by culture.

By the end of the third and fourth months of therapy, cultures were positive in only 9 of the 16 cases. Sensitivity tests of organisms from these 9 positive cultures indicated that all of them had developed a ten-fold or greater increase in resistance. Five of these 9 strains developed a 10- to 50-fold increase in resistance; the remaining 4 strains developed a 100 to greater than 2,000-fold increase in resistance.

It was not possible with this small group of patients to demonstrate conclusively a correlation between resistance to streptomycin and clinical course under therapy.

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PLASMA VOLUME, TOTAL CIRCULATING PROTEIN, AND "AVAILABLE FLUID" ABNORMALITIES IN PREECLAMPSIA AND ECLAMPSIA¹

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It has long been recognized that the red blood count may be abnormally high in eclampsia (1). Dieckmann (2) believed that the elevation of hematocrit was consequent upon a diminution in plasma volume, but this concept has not received general recognition.

In contrast to the elevation of hematocrit Strauss (3) and others (4, 5) stressed the low plasma albumin concentration which they believed to play a part in the oliguria, water retention and edema. If the plasma volume is indeed low, as claimed by Dieckmann, the combination of a low plasma volume and low plasma protein concentration would result in a marked deficiency of total circulating protein. Many of the clinical features of severe toxemia such as the edema, albuminuria and oliguria suggest that this may well be the case. With these considerations in mind we undertook to investigate the changes in plasma volume, total circulating protein and "available fluid" (thiocyanate space) associated with preeclampsia and eclampsia as compared to normal pregnancy.

MATERIALS AND METHODS

The patients with toxemia were studied under as nearly basal conditions as possible on the wards of the obstetrical service of the Massachusetts Memorial Hospitals. All of these patients were diagnosed clinically as having eclampsia or preeclampsia. In every case the pathological manifestations began in the last trimester of pregnancy. All cases diagnosed as having preeclampsia exhibited hypertension, edema and albuminuria. Cases of preeclampsia were called severe when in addition they exhibited oliguria, visual disturbances and/or epigastric pain.

Two control groups were used in this study. One was comprised of seven cases in the last trimester of normal pregnancy. Four of these patients manifested clinical edema of the face, hands and feet but were otherwise

normal. The other control group consisted of six normal nonpregnant females (nurses and laboratory technicians). In all cases the subjects of both control groups were fasting at the time of the examination, and were resting in the supine position for at least one-half hour prior to the test.

Plasma volume and "available fluid" determinations were carried out with the dye T-1824 and a 5 per cent solution of sodium thiocyanate, respectively, as detailed in a previous communication (6). The "available fluid" volume as measured by this method includes all of the fluid spaces to which thiocyanate is distributed at equilibrium, including the plasma volume as well as the amount that enters the red blood cells. In the pregnant individual thiocyanate in all probability passes the placental membrane, but this fact does not nullify comparative studies between toxemic and normal pregnancy in the same stage of gestation. Total protein was determined from the plasma specific gravity by the method of Barbour and Hamilton (7), as modified by Weech (8).

RESULTS

"Available fluid"

The "available fluid" volume was highest in the severe preeclamptic and eclamptic group of patients, the mean being 321 ml. per kilo body weight and the range 291 to 355 ml. per kilo (Table I). The patients with normal pregnancy exhibited a mean "available fluid" volume of 282 ml. per kilo, range 235 to 315 ml. per kilo, while the nonpregnant normal females showed an average of 245 ml. per kilo body weight and a range of 210 to 267 ml. per kilo (Table II and III). Thus, in general, the greatest volumes of "available fluid" were encountered in the toxemic patients and the smallest volumes in the nonpregnant individuals, although there was considerable overlapping between the toxemic group and the group with normal pregnancy. The "available fluid" volume was well correlated with the degree of edema.

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TABLE I

Plasma volume, total circulating protein and "available fluid" volume in severe preeclampsia and eclampsia

Case	Time from delivery	Ht.	Wt.	Plasma volume		Hematocrit value	Total protein	Total circulating protein		"Available fluid"		Ratio
		cm.	kilo	ml.	ml./kilo			gms.	gms./kilo	ml.	ml./kilo	
Eclampsia 1—L. H.	12 hrs. pre	170	93.2	3,440	37.0	39	5.3	182	2.0	27,100	291	7.9
	3 days post					37	5.4			22,800		
	14 days post		79	3,400	43.0	33	6.1	207	2.6	21,800	275	6.4
2—M. S.	2 days pre	165	72.2	3,560	49.3	38	6.2	220	3.0	24,200	335	6.8
	½ day pre		73	5,320	72.9	30	5.9	314	4.3	26,400	361	5.0
	(1,750 ml. plasma)*											
	3 days post		66	3,360	49.4	29	5.5	185	2.8	22,400	339	6.8
	10 days post		59.4	2,270	38.2	38	6.5	147	2.5	15,200	254	6.7
Pre-eclampsia severe 3—E. H.	1 day pre	165	62.9	2,860	45.4	39	4.25	122	1.9	22,400	355	7.8
	(500 ml. plasma)*											
	½ day post		54	2,800	51.8	35						
	4 days post		49	2,760	56.3	33	5.3	146	3.0	15,450	315	5.4
	11 days post		46.4	2,480	53.4	34	5.5	136	2.9	13,900	299	5.6
	35 days post		48.1	2,500	51.9	38	5.5	137	2.8	15,200	315	5.6
4—V. P.	6 hrs. pre	169	68.6	3,440	50.1	37	4.7	162	2.3	21,300	310	6.2
	10 days post		59	2,420	41	39	6.3	152	2.6	16,100	273	6.6
5—E. G.	1 day pre	160	47.7	1,927	40.4	45	5.2	100	2.1	15,025	315	7.8
Mean					44.3				2.2		321	7.3

* Intravenous administration of citrated plasma between the first and second plasma volume determinations.

Plasma volume

The plasma volume determinations for the six normal nonpregnant females agreed well with those of other investigators for a similar group (9, 10), the range being 38.3 ml. to 54.3 ml. per

kilo body weight with a mean of 44.8 ml. per kilo (Table II). The plasma volume levels for the normal pregnancy control group were much higher than the nonpregnant controls, the mean being 59.1 ml. per kilo and the range 47.0 to 70.5 ml. per

TABLE II

Plasma volume, total circulating protein and "available fluid" volume in normal nonpregnant females

Subject	Ht.	Wt.	Plasma volume		Hematocrit value	Total protein	Total circulation protein		"Available fluid"		Ratio
	cm.	kilo	ml.	ml./kilo			gms.	gms./kilo	ml.	ml./kilo	
M. F.	161	57.7	2,300	39.9	36	6.85	157	2.7	12,080	210	5.2
E. H.	167	46.8	2,540	54.3	38	6.6	168	3.5	12,450	267	4.9
C. B.	172	63.0	2,780	44.1	42	7.0	195	3.1	14,900	236	5.4
G. M.	165	62.1	2,375	38.3	42	6.4	152	2.4	14,000	226	5.9
V. N.	159	59.1	2,620	44.1	35	5.9	155	2.6	15,400	261	5.9
A. C.	172	60.0	2,910	48.5	38	5.5	160	2.7	15,800	263	5.4
Mean				44.8				2.8		245	5.5

TABLE III

Plasma volume, total circulating protein and "available fluid" volume in the third trimester of normal pregnancy

Patient	Ht.	Wt.	Edema	Plasma volume		Hema- to- crit value	Total protein	Total circulating protein		"Available fluid"		Ratio
				ml.	ml./kilo			gms.	gms./kilo	ml.	ml./kilo	
M. J.	160	63.5	+	4,180	64.2	34	6.8	284	4.4	19,700	310	4.7
C. G.	165	91.0	+	4,750	52.5	35	6.25	296	3.3	21,400	237	4.5
D. V.	167	69.0	+	4,860	70.5	32	6.4	311	4.5	21,700	315	4.5
M. K.	162	54.2	0	3,595	66.0	35	5.4	194	3.6	16,000	295	4.5
M. B.	171	81.5	+	3,890	47.8	36	6.1	237	2.9	21,100	259	5.4
M. L.	160	57.5	0	3,780	66.0	32	5.7	215	3.7	18,600	323	4.9
E. G.	166	105.0	0	4,950	47.0	40	6.1	305	2.9	24,800	235	5.2
Mean					59.1				3.6		282	4.8

kilo (Table III). In contrast, the five patients diagnosed clinically as having either severe pre-eclampsia or eclampsia exhibited a mean plasma volume of 44.3 ml. per kilo, range 37 to 50.1 ml. per kilo. Thus, in respect to plasma volume the patients with severe toxemias of late pregnancy more nearly resembled the nonpregnant controls than the pregnant controls.

Although the cases of severe toxemia in general exhibited lower plasma volumes in respect to body weight than patients with normal pregnancy there was some overlapping of individual cases (Figure 1). The plasma volume in respect to height or surface area still showed overlapping of the various groups.

However, when the plasma volume was considered in relation to the total extracellular fluid

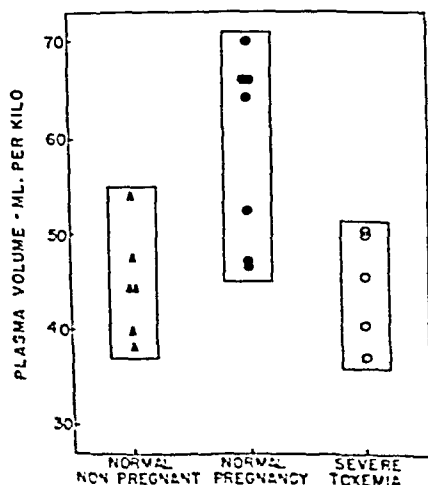


FIG. 1. SHOWING THAT THE PLASMA VOLUME IN RELATION TO BODY WEIGHT IS REDUCED IN SEVERE TOXEMIA AS COMPARED TO NORMAL PREGNANCY BUT THAT SOME OVERLAPPING OF INDIVIDUAL CASES OCCURS

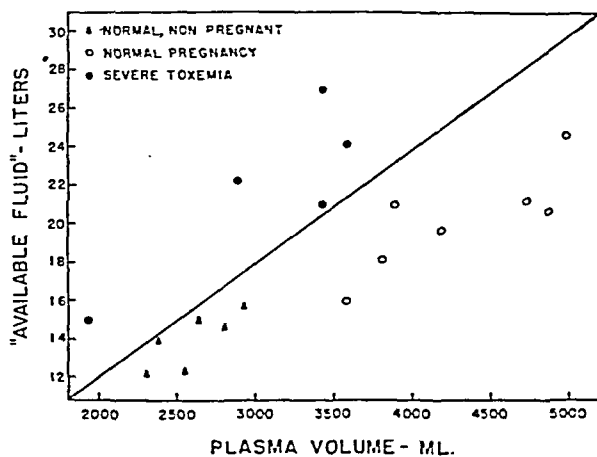


FIG. 2. SHOWING THAT THE RELATIONSHIP BETWEEN PLASMA VOLUME AND "AVAILABLE FLUID" RESULTS IN A CLEAR CUT DIFFERENTIATION BETWEEN THE SEVERE PREECLAMPTIC AND ECLAMPTIC PATIENTS AS COMPARED TO THE NORMAL NONPREGNANT AND THE NORMAL PREGNANT INDIVIDUALS

space as measured by thiocyanate, all patients with severe toxemia fell into a group distinct from the normal nonpregnant and the normal pregnant individuals (Figure 2). In normal pregnancy an increase in "available fluid" volume was balanced by a commensurate increase in plasma volume; the ratio of "available fluid" volume to plasma volume remained the same or was lower than the normal nonpregnant state. But in severe toxemia the relationship was no longer normal due to a failure of plasma volume to keep pace with the increasing extracellular fluid space. The distinguishing criteria of severe toxemia, therefore, lay in the ratio between the comparative volumes of the plasma and the total extracellular fluid, as measured by thiocyanate.

Additional evidence for a deficiency in plasma volume in the toxemias of late pregnancy was provided by Cases 1 and 3 of Table I. In normal pregnancy there is a marked increase in plasma volume during the period of gestation and a pronounced fall following delivery (11). Case 1 (Table I), an eclamptic, in contrast to patients with normal pregnancy, exhibited approximately the same plasma volume two weeks following delivery as she had prior to delivery. Similarly, the plasma volume of Case 3, who had severe preeclampsia, was determined 35 days following delivery. At this time she had experienced a normal menstrual period and was not nursing her child. Using this value as the nonpregnant basal level it was determined that the absolute plasma volume had increased only 13 per cent by the end of gestation, and that the plasma volume in terms of body weight had actually decreased by 13 per cent.

Total circulating protein

The total circulating protein was increased in normal pregnancy over that observed in the normal nonpregnant group. The mean for the nonpregnant control group was 2.8 gms. per kilo body weight, range 2.4 to 3.5, while in the group with normal pregnancy the mean was 3.6 gms. per kilo, range 2.9 to 4.5. This increase was due to an enlargement of plasma volume rather than to an increased plasma protein concentration. The

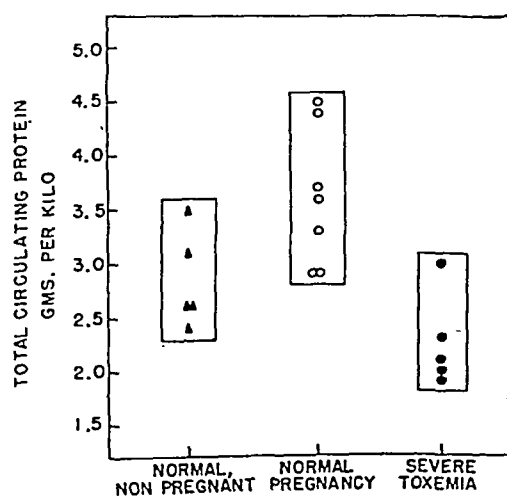


FIG. 3. SHOWING THAT THE TOTAL CIRCULATING PROTEIN IN RELATION TO BODY WEIGHT IS REDUCED IN SEVERE TOXEMIA AS COMPARED TO NORMAL PREGNANCY BUT THAT SOME OVERLAPPING OF INDIVIDUAL CASES OCCURS

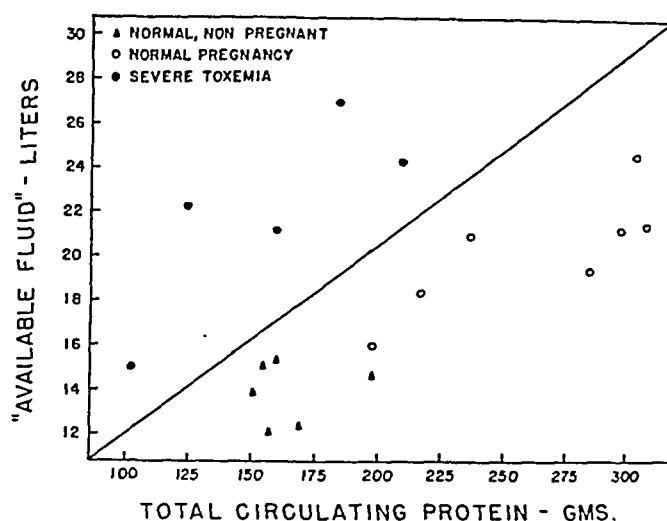


FIG. 4. SHOWING THAT THE RELATIONSHIP BETWEEN TOTAL CIRCULATING PROTEIN AND "AVAILABLE FLUID" RESULTS IN A CLEAR CUT DIFFERENTIATION BETWEEN THE SEVERE PREECLAMPTIC AND ECLAMPTIC PATIENTS AS COMPARED TO THE NORMAL NONPREGNANT AND THE NORMAL PREGNANT INDIVIDUALS

total circulating protein tended to be reduced in the cases of severe preeclampsia and eclampsia, the mean being 2.2 gms. per kilo body weight and the range 1.9 to 3.0. The reduction in total circulating protein was due in part to a reduction in plasma volume and in part to a reduced plasma protein concentration.

When the total circulating protein values were plotted in terms of body weight, as in the case of plasma volume, there was some overlapping of individual cases between the toxic patients and the patients with normal pregnancy (Figure 3). However, as in the case of plasma volume, a clear distinction between the two groups became apparent in the relationship between total circulating protein and "thiocyanate space" (Figure 4).

Mild preeclampsia

The cases classified on clinical grounds as mild preeclampsia did not demonstrate the above-mentioned changes to such a striking degree (Table IV). These patients manifested edema, hypertension and albuminuria, but did not have the oliguria, headaches, visual scotomata or epigastric pain associated with the more severe forms. They belonged to the category designated as preeclampsia, grade I. The mean plasma volume for these four cases was 55.8 ml. per kilo, the average total circulating protein was 3.0 gms. per kilo and

TABLE IV

Plasma volume, total circulating protein and "available fluid" volume in mild preeclampsia

	Time from delivery	Ht.	Wt.	Plasma volume		Hematocrit value	Total protein		Total circulating protein		"Available fluid"		Ratio
		cm.	kilo	ml.	ml./kilo		gms. %	gms.	gms./kilo	ml.	ml./kilo	cr. fl./P.V.	
1—P. S.	7 days pre	155	103	4,750	46.0	39	5.7	271	2.6	27,030	262	5.7	
	4 days pre		103	4,890	47.4	39	6.3	308	3.0	26,320	255	5.4	
	1 day pre		98	4,330	46.3	39	5.7	247	2.5	25,640	274	5.9	
	2 days post		91	3,600	40.0	39	6.1	220	2.4	23,800	261	6.5	
	9 days post		90	3,500	38.8	40	6.8	238	2.6	22,200	246	6.3	
2—V. F.	1 day pre	168	75.5	4,920	65.2	37	5.5	271	3.6				
	5 days post		59	2,408	40.8	42	5.9	142	2.4				
3—M. C.	1 day pre	165	71.4	4,320	60.5	31	5.1	220	3.1	22,050	307	5.1	
	8 days post		64.5	3,230	50.0	25	5.9	191	2.9	19,600	304	6.0	
4—E. L.	2 wks. pre	165	81.5	4,190	51.5	37	5.7	238	2.9	23,400	287	5.6	
Mean					55.8				3.0		285	5.3	

the ratio $\frac{\text{"available fluid"}}{\text{plasma volume}}$ was 5.3 in the three cases in which it was determined. It is to be noted that in respect to plasma volume and total circulating protein the first case of this mild group falls into the same category as the severe preeclampsias while the remaining cases fall into the normal group. It would appear that patients with the clinical diagnosis of mild preeclampsia belong to a heterogeneous group in which fluid volume relationships may be normal or slightly abnormal.

The hematocrit value tended to be higher than that usually seen in late pregnancy ranging between 37 and 45 in the group with severe toxemia. However, a close correlation between the degree of depression of plasma volume and the degree of elevation of the hematocrit value was not demonstrated.

DISCUSSION

The disturbed relationship observed between total circulating protein and plasma volume on the one hand and the "available fluid" volume on the other brings into consideration the forces that play a part in membrane equilibria. The following hypothesis is suggested.

In normal pregnancy when edema occurs interstitial fluid pressure rises. This rise upsets the balance between filtration and osmotic pressure so that water and crystalloids move into the plasma, and plasma dilution takes place. However, in normal pregnancy there is, apparently, a

response on the part of the organism to add excess protein to the circulation. It will be noted in Figure 4 that those cases of normal pregnancy with the largest "available fluid" volumes also showed the greatest increases in total circulating protein. By the addition of this excess protein plasma oncotic pressure is maintained. As a result despite the edema the relationship of plasma to "available fluid" volume remains approximately the same as in the normal nonpregnant state. Figure 2 reveals such a straight line relationship between the normal nonpregnant individual and the normal pregnant individual.

In contrast to the edematous patient with normal pregnancy, the severely preeclamptic or eclamptic patient who develops edema is apparently unable to maintain the needed excess of circulating protein (Figure 4). Because of this failure, as plasma dilution occurs, plasma protein concentration, and hence, plasma oncotic pressure fall. Strauss has shown this fall in plasma oncotic pressure in preeclamptic and eclamptic patients by actual measurement (3). The result is a relative decrease in plasma volume and a deviation from the normal in the relationship between "available fluid" volume and plasma volume (Figure 2).

The observed abnormalities in fluid volume relationships in late toxemia are in all probability the result rather than the cause of the disease. Nevertheless, it is possible that these alterations may play an important role in certain of its more serious manifestations, such as the oliguria, the

cerebral edema (12, 13, 14) and the occasional termination in vascular collapse (15).

The results of this investigation have delineated two groups of preeclamptic patients: the severe cases who have headaches, visual scotomata, epigastric pain, oliguria, a diminution in plasma volume, total circulating protein, edema, and an elevation of the $\frac{\text{"available fluid"}}{\text{plasma volume}}$ ratio. Cases in

the second group exhibit edema, albuminuria and hypertension, but have only mild symptoms referable to the central nervous system and do not tend to develop oliguria. Cases in this latter group may show normal or only slightly abnormal patterns of fluid distribution. These observations are in exact agreement with those of Dieckmann (2).

Additional evidence for the separation of preeclamptic patients into two groups is provided by Boyd (16). He observed that in some of these patients the blood lipid partition pattern was characteristic of eclampsia, while in others the distribution of blood lipids was similar to that observed in normal pregnancy. Boyd suggested that the cases of preeclampsia with an abnormal blood lipid partition are in reality eclampsia without convulsions, while the remaining cases with normal blood lipids are not literally preeclamptic at all. Our observations lend support to the hypothesis that severe preeclampsia and eclampsia are the same disease while many cases of mild preeclampsia belong in a different category. However, because of the relatively small number of cases we have studied and the difficulty in the clinical differentiation between mild and severe preeclampsia these results are to be regarded as suggestive rather than conclusive.

Although it is well recognized that eclampsia may occur in the well-nourished, it is of interest that all of the patients with severe preeclampsia and eclampsia gave histories of poor protein intake. This observation is in agreement with that of Strauss (3) and others (5, 17, 18). The importance of an adequate protein intake in the prophylaxis of the eclamptic toxemias of pregnancy would appear to merit further investigation.

SUMMARY AND CONCLUSIONS

1. Changes in plasma volume, total circulating protein and "available fluid" volume were followed in 4 cases of mild preeclampsia, 3 cases of

severe preeclampsia and 2 cases of eclampsia; and were compared to the changes observed in 7 cases of normal pregnancy and 6 normal nonpregnant females.

2. The cases of severe preeclampsia and eclampsia revealed an abnormal reduction in plasma volume, and total circulating protein, in respect to "available fluid" volume.

3. Cases classified clinically as mild preeclampsia were made up of a heterogeneous group, some of which showed slight changes in fluid and protein distribution characteristic of eclampsia, others exhibiting changes typical of normal pregnancy.

4. It is suggested on the basis of the admittedly small series of cases that severe preeclampsia and eclampsia are characterized by an abnormal fluid distribution in which a deficiency of total circulating protein may play an important role.

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CARDIOVASCULAR REACTIONS TO EMOTIONAL STIMULI. EFFECT ON THE CARDIAC OUTPUT, ARTERIOVENOUS OXYGEN DIFFERENCE, ARTERIAL PRESSURE, AND PERIPHERAL RESISTANCE¹

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INTRODUCTION

Emotional disturbances may have a profound effect on the circulation, causing changes in the heart rate, cardiac output (1 and 2), blood pressure, tone of peripheral vessels, and the electrocardiogram (3). This is particularly true of the emotional state which may develop in persons who find themselves in a hazardous situation. For present purposes this emotional state is termed "anxiety," although it is recognized that other reactions such as resentment or anger may also occur, depending upon the individual and the circumstances. In any study on unanesthetized human subjects, changes produced by anxiety may mask completely the physiologic or pharmacologic effects which are obvious in a relaxed subject. In the interpretation of experimental data, objective criteria by which it can be established, or even suspected, that anxiety is having an effect on cardiovascular function are useful. It is also of importance to clinical medicine to have further information concerning the degree to which anxiety may affect certain aspects of cardiovascular function and on the mechanisms by which the effects of organic cardiovascular disease may be simulated or intensified by anxiety. It is the purpose of the present report to make a contribution in both of these directions.

METHODS

The interpretation of data relating anxiety to circulatory changes is handicapped by the absence of a reliable objective means of measuring the degree of anxiety induced or even of detecting the presence of anxiety at all. In judging variation in response to anxiety, it is difficult to distinguish between variation in the intensity of the stimulus and in the reactivity of different subjects. In

the present study, it has been necessary simply to place reliance on the statement of the subject that he had a sensation of anxiety when the first set of measurements was made and that the sensation had abated or was absent in the control period. It was not feasible to define the emotional state of each subject more exactly than this. An attempt was made to reduce circulatory fluctuations due to causes other than anxiety by conducting control studies under conditions which were otherwise as similar as possible. All subjects were recumbent when studied.

Two groups of subjects were studied. The first group consisted of 23 unselected healthy medical students, ranging in age from 20 to 25 years. Anxiety existed in these subjects because of the imminence of an important academic oral examination. Cardiac output was measured on the Nickerson ballistocardiograph (4) immediately before the examination of each student and again 24 hours after the examination, when anxiety was presumably absent or much abated. Blood pressure by the auscultatory method and pulse rate were determined at the same times. Mean pressure was estimated by adding $\frac{1}{3}$ the pulse pressure to the diastolic pressure. In each case, measurements were made at least two hours after the last meal and following a rest period of approximately 20 minutes, the first half of which was spent sitting quietly and the second half recumbent on the ballistocardiograph table. Intercurrent illness was ruled out by a brief history.

The second group of subjects consisted of hospital and clinic patients. In this group cardiac outputs were determined by the Fick method following intracardiac catheterization (1, 5, and 6). Direct brachial arterial, intracardiac, and pulmonary arterial pressures were recorded by means of a Hamilton manometer, and mean pressures were determined by planimetric integration. Anxiety was first induced by the statement that the procedure, though necessary, involved some risk and by the simulation of a tense and watchful attitude on the part of the observers themselves. The patient was then told that all danger was past, and that the response to the test had been excellent. Occasionally seconal was given in doses of 0.1 to 0.2 gram. In these patients observations were made about one hour apart at a single sitting. In some cases observations were made during light leg exercise with the patient in a recumbent position.

In all cases the peripheral resistance (7) was expressed as the ratio of mean arterial pressure in mm. Hg to the cardiac index. This is a function proportional to the

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absolute value of the peripheral resistance corrected for the surface area to permit ready comparison of peripheral resistance in persons of different body size.

RESULTS

Anxiety in normal young males

The data obtained on the group of 23 students immediately before examination and 24 hours later are summarized in Figure 1. In the pre-examination, or anxious, period each of these subjects admitted to a feeling of tenseness. Subjective observations included most frequently warmth of the face, coldness and sweating of the hands, axillary sweating, palpitation, general shakiness, epigastric uneasiness, and anorexia. There was one report of frank diarrhea and one of frequent urination. Objectively malar flush and clammy hands were most prominent. Tremor of the voice was noted occasionally. In the subsequent control period none of these manifestations of anxiety was present. In general, the attitude was one of relief and satisfaction. There had been no academic failures in this group. Choosing the control observations on each subject as a reference point, the group as a whole during the period of anxiety showed an increase of 10% in the mean arterial pressure (from 90 to 99 mm. Hg), an increase of

27% in the heart rate (from 70 to 89 beats/min.), an increase of 16% in the stroke volume (from 112 to 130 cc.), an increase of 48% in the cardiac index (from 4.2 to 6.2 liters/min./sq.m.), and a fall of 23% in the peripheral resistance (from 22 to 17 mm. Hg/liters/min./sq.m.). There was great variation in the amount of change in different individuals between the composed and anxious states. This was particularly true of the cardiac index, as illustrated by Figure 1. In Figure 2 is shown the relation between change in cardiac index and that in peripheral resistance during the anxious state. For each subject the control period is represented by the origin. It will be observed that there is a tendency for correlation between the degree of increase in cardiac index and the degree of fall in peripheral resistance in the anxious state. In general, the greater the increase in cardiac index, the greater the fall in peripheral resistance. In Figure 3, the change in cardiac index in the anxious state is similarly plotted against the change in mean arterial pressure. It is evident that the mean pressure either remained unchanged or increased during the anxious state, but no correlation is evident between the extent of change in the mean pressure and that in the cardiac index.

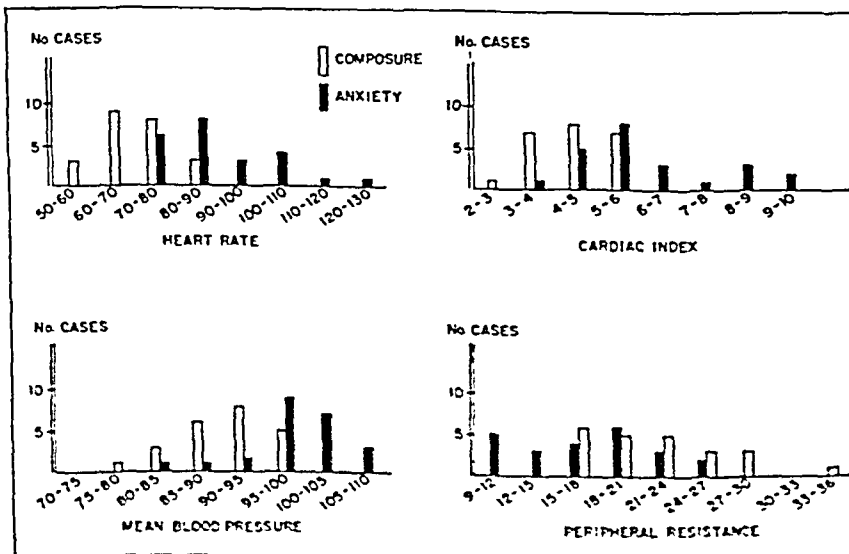


FIG. 1. EFFECT OF ANXIETY ON VARIOUS CIRCULATORY VALUES IN 23 NORMAL MALE STUDENTS

Heart rate is expressed in beats/min.; cardiac index in liters/min./sq.m.; mean blood pressure in mm. Hg; and peripheral resistance in mm. Hg/liters/min./sq.m.

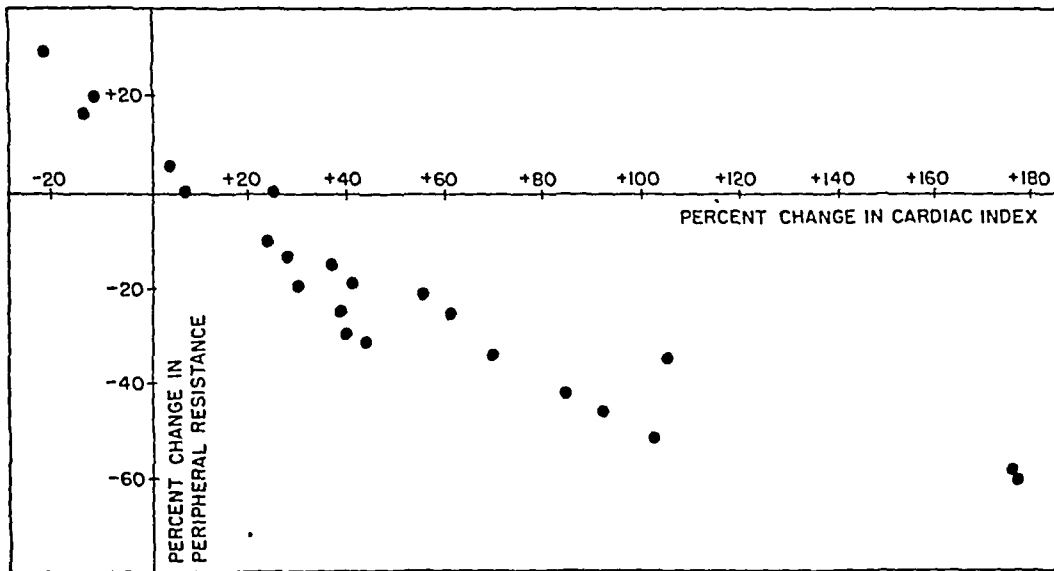


FIG. 2. RELATION OF CHANGE IN CARDIAC INDEX TO CHANGE IN PERIPHERAL RESISTANCE DURING THE ANXIOUS STATE IN 23 NORMAL MALE STUDENTS

The control values for each student during composure are represented by the origin.

For the group, as a whole, the effect of anxiety was to produce a large increase in cardiac index, a large drop in peripheral resistance, and a relatively small rise in mean arterial pressure. The increase in arterial pressure was on the basis of an increased cardiac index rather than an overall vasoconstriction. In four cases, however, the anxious state was associated with a slight to moderate increase in peripheral resistance, and it was primarily or entirely on this basis that the increase in blood pressure occurred. These four cases showed no increase or a slight fall in cardiac index during the anxious state. In three sub-

jects who showed increases of cardiac index between 35 and 56% during the anxious state, there were large moment-to-moment fluctuations in cardiac index as the result of changes in both stroke volume and heart rate. An average value from several determinations has been given in each case.

Observations on hospital and clinic patients

The second group of subjects, in whom observations were made during intracardiac catheterization, consisted of 12 hospital and clinic patients.

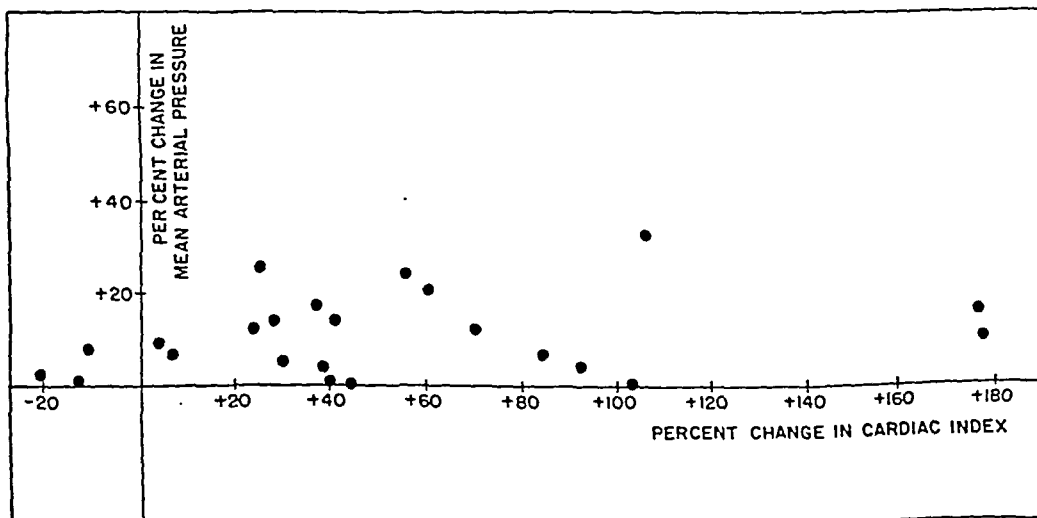


FIG. 3. RELATION OF CHANGE IN CARDIAC INDEX TO CHANGE IN MEAN ARTERIAL PRESSURE DURING THE ANXIOUS STATE IN 23 NORMAL MALE STUDENTS

TABLE I

Patient	Age	Sex	Diagnosis	State	Heart rate	Atrial press.	Mean pulm. art. press.	Periph. art. press.		A-V O ₂ diff.	O ₂ consump.	Cardiac index	Periph. resist.
								Sys. dias.	Mean				
						mm. Hg	mm. Hg	mm. Hg	mm. Hg	vol. %	cc./min./sq. m.	l./min./sq. m.	mm. Hg./l./min./sq. m.
1. G. D.	30	M	Asymptomatic CNS syphilis	Anxiety	73		12	140/88*	105	2.9	141	4.9	21.4
				Exercise	82		14	110/80*	90	6.0	314	5.2	17.3
2. W. H.	15	M	Latent syphilis	Anxiety	62	4	12	95/53	68	3.1	177	5.7	11.9
				Exercise	72	5	14	119/67	87	5.8	387	6.7	13.0
3. T. C.	44	M	Emphysema, hyperthyroidism	Anxiety	114	0	18	133/74	92	3.4	220	6.5	14.1
				Exercise	114		22	150/79	98	6.5	285	4.4	22.2
4. C. N.	35	F	Methemoglobinemia	Anxiety	89	4	18	128/70*	89	3.1	205	6.7	13.4
				Exercise	80		20			4.5	284	6.4	
5. F. J.	24	M	A-V fistula	Anxiety	83	2	14	133/70	91	3.1	179	5.7	15.9
				Composure	72	2	13	132/70	91	3.8	172	4.5	20.2
6. S. C.	44	M	Hypertension	Anxiety	79		16	203/102	137	2.6	171	6.7	20.4
				Composure	75		9	191/115	142	4.3	163	3.8	37.4
7. E. H.	35	F	Hypertension	Anxiety	60		8	202/108	153	5.1	127	2.5	61.2
				Composure	66	3	10	190/113	142	4.4	123	2.8	50.7
8. O. T.	55	F	Aortic insufficiency, syphilis	Anxiety	78	0	6	146/50	84	5.4	139	2.6	32.3
				Composure	77		10	143/49	84	3.0	126	4.3	19.5
				Exercise	100	3	19	182/72	120	4.6	413	9.0	13.3
9. E. C.	21	F	Convalescent pneumonia	Anxiety	82		9	105/65	80	4.4	142	3.2	25.0
				Composure	92	1	9	112/72	84	4.1	126	3.1	27.0
				Exercise						6.8	285	4.2	
10. C. B.	41	M	Congestive failure, etiology unknown	Anxiety						8.3	170	2.1	
				Composure						6.2	153	2.5	
11. C. Br.	54	F	Congestive failure, syphilitic AI	Anxiety	78		15	179/57	103	7.5	138	1.8	57.3
				Composure	68	0	12	180/53	97	6.0	122	2.0	48.5
12. G. S.	47	M	Congestive failure, syphilitic AI	Anxiety	77		37	153/51	87	5.7	148	2.1	41.5
				Composure	68	-2	25	149/55	88	6.7	120	2.2	40.0
				Exercise	84	5	54	193/62	108	7.0	210	3.0	36.0

* Auscultatory.

The data obtained on these subjects are presented in Table I. In most cases, an attempt was made to induce anxiety during the early part of the procedure. In the remainder, anxiety was spontaneous. All subjects admitted to a feeling of tenseness or apprehension. All subjects are included in whom an attempt was made to induce anxiety during the course of this study.

The relation between rate of oxygen consumption and cardiac index is shown in Figure 4. In this figure, the rectangle indicates the range covered by 18 basal subjects observed by Stead *et al.* (1). The area inclosed by the two straight lines drawn to the extremities of the rectangle indicates the range covered by subjects with a nor-

mal cardiovascular system studied by Hickam and Cargill (8) during rest and exercise. It is believed that points falling within the region so inclosed are indicative of a normal response of the cardiac index to the body oxygen requirement. Points to the right of the area represent an unusually high cardiac index, and those to the left indicate an unusually low cardiac index in proportion to the oxygen consumption.

For convenience in presentation, the present group is subdivided according to the procedure followed and the condition of the patient.

Anxiety followed by exercise. In cases 1, 2, 3, and 4, observations were made during the anxious state and then during a period of light to

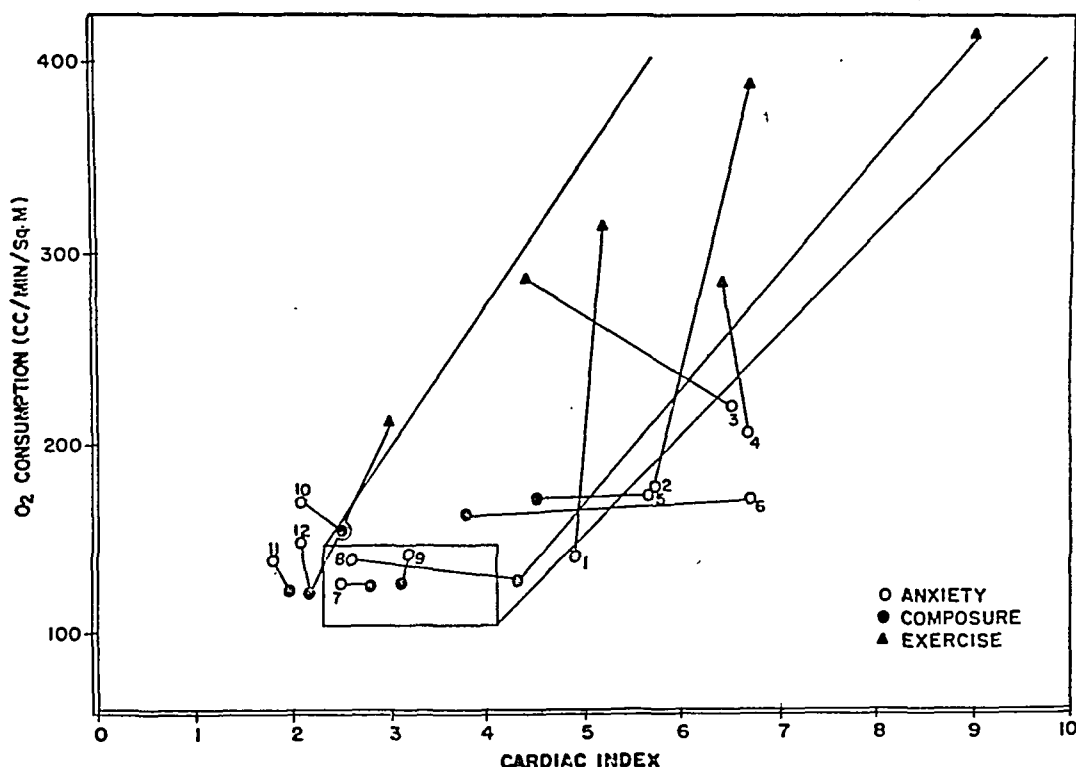


FIG. 4. RELATION OF CARDIAC INDEX TO OXYGEN CONSUMPTION IN SUBJECTS IN STATES OF ANXIETY, COMPOSURE, AND MUSCULAR EXERCISE

Connecting lines identify points belonging to the same subject. Rectangle covers normal basal range—lines from extremities of rectangle cover range of values exhibited by normal persons during muscular exercise.

exercise which followed immediately. Patients 1 and 2 had asymptomatic syphilis, without evidence of cardiovascular disease. Patient 3 had pulmonary emphysema. Because of a consistent elevation of the BMR hyperthyroidism was suspected. Patient 4 complained of weakness and cyanosis and was found to have methemoglobinemia, apparently on the basis of ingestion of drugs containing acetanilid. Patients 3 and 4 both had dyspnea on moderate exertion, but in neither case was there any cardiac enlargement. All four patients, as is evident in Figure 4, had a cardiac index during the anxious state which was abnormally high for the rate of oxygen consumption. During the subsequent exercise period, however, the relation between cardiac index and oxygen consumption shifted to the normal range. In patients 1 and 2 exercise resulted in a very slight increase of cardiac output over the previous level, while in patients 3 and 4 the increased oxygen consumption caused by exercise was associated with an actual fall in cardiac output.

Anxiety followed by composure. In cases 5, 6, 7, 8, and 9 the initial observations, which were

made while the patients were considered to be in an anxious state, were followed by a period of rest and reassurance. Second in doses of 0.1 to 0.2 gram was given by mouth. After an hour observations were repeated. These patients were all in the post-absorptive state and had been at complete physical rest for at least half an hour before the first observations were made. Case 5 had a traumatic A-V fistula; 6 and 7 had essential hypertension; 8 had syphilitic aortic regurgitation; 9 was convalescent from pneumonia. There was no evidence of cardiac decompensation in any of these patients.

The behavior of cases 5 and 6 was similar to that of most of the normal students. As indicated in Figure 4 the cardiac index was abnormally high in proportion to the rate of oxygen consumption during the period of anxiety. On composure, the cardiac index fell, assuming a normal relation to the oxygen consumption. During anxiety the peripheral resistance was lower than during composure. The mean arterial pressure showed little change.

The behavior of cases 7 and 8 was opposite to

that expected. In both cases the peripheral resistance was higher during the period of anxiety than during composure. During anxiety patient 7, who had essential hypertension, had a moderate increase of mean arterial pressure over the control level, but no important change in cardiac index or oxygen consumption. During anxiety patient 8 had no change in pressure but the cardiac index was well below the control level. In this patient oxygen consumption during anxiety was 13% above the control level. Both patients stated that they experienced some anxiety during the first period of observation and relative composure during the second. It is primarily on the basis of this statement that their cardiovascular reactions are assigned respectively to states of anxiety and composure. The reaction of these patients appears to be similar to that of the four students who, during anxiety, had a rise in peripheral resistance and no change or a fall in cardiac index.

Patient 9 showed no important change in peripheral resistance or cardiac index between the two periods, but the rate of oxygen consumption was 10% higher during anxiety than during composure.

Anxiety in patients with cardiac decompensation. Patients 10, 11, and 12 had recently had congestive heart failure, of unknown etiology in case 10, and the result of syphilitic aortic regurgitation in the other two. All had improved to the extent of being comfortable while flat in bed. As indicated in Figure 4, the cardiac output of these patients was abnormally low for the rate of oxygen

consumption. In the period of anxiety the rate of oxygen consumption was increased by 11, 14, and 23%, respectively, over that in the period of composure. There were, however, no substantial changes in cardiac output or peripheral resistance between the two periods. The data on the subsequent exercise period of patient 12 indicate that his ability to increase his cardiac output was extremely limited, as is typical of persons with cardiac decompensation. Mean pulmonary arterial pressures were measured in patients 11 and 12. In patient 11, in whom the pulmonary pressure was not abnormally elevated, there was no substantial change as the result of anxiety. This was generally true for the patients without cardiac decompensation. In case 12, however, in whom the pulmonary pressure was abnormally elevated during the period of composure, there was a 50% increase in pressure during the period of anxiety. In the exercise period there was a 100% increase in pressure. Pulmonary pressure tracings from this patient are reproduced in Figure 5.

DISCUSSION

In attempting to compare values for cardiac output in different subjects, it is customary to make observations at complete physical rest. It is obvious that physical rest does not mean emotional relaxation and that because of this fact, widely divergent values for cardiac output are obtained even when the figures are reduced to the same surface area. The scatter of the values was re-

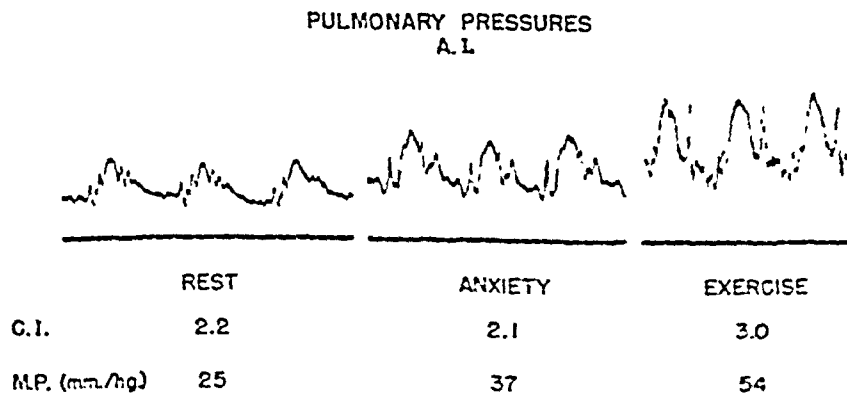


FIG. 5. INFLUENCE OF ANXIETY AND MUSCULAR EXERCISE ON PULMONARY ARTERIAL PRESSURE IN A PATIENT WITH CONGESTIVE HEART FAILURE RESULTING FROM AORTIC INSUFFICIENCY

duced by light exercise. It appears that resting values are by no means the most constant values.

The data recorded here, together with that reported elsewhere, demonstrate the great variability in the response of the circulation to anxiety. This variability is qualitative as well as quantitative. Stimuli from the emotional content of thought may pass out over both sympathetic and parasympathetic portions of the autonomic nervous system. The autonomic nervous system may affect the heart by altering its rate and output, and the peripheral vessels by causing vasoconstriction or vasodilatation. In addition to the direct effects on the circulation from such stimuli, there may be indirect effects. For example, a lowering of the peripheral resistance causes an immediate rise in cardiac output, the mechanism of which has not been determined. At present, there is no way of determining which changes in the circulation result from stimuli from the emotional content of thought acting directly on the heart and which are secondary to changes in the peripheral vessels with secondary effect on the heart.

In most subjects the cardiovascular effects of anxiety are similar to those produced by small doses of epinephrine (9). The heart rate and cardiac output are increased. There is a moderate elevation of blood pressure, but the peripheral resistance (ratio of mean arterial pressure to cardiac output) is decreased, apparently indicating a predominant dilatation of the vascular bed. The vessels of the hands and feet are constricted. There is a moderate increase in rate of oxygen consumption.

In certain subjects, severe anxiety may be attended by circulatory collapse (10). In such cases the overall peripheral resistance is decreased, as in the type of reaction described above, but the heart rate is slowed and the cardiac output does not increase in the expected fashion as the peripheral resistance decreases. There results a profound fall of blood pressure. Both types of reactions have been observed in the same subject at different times. Both reactions appear to have in common an overall decrease in vascular tone, together with evident vasoconstriction in certain regions, such as the hands and feet. In the first type of reaction, however, the general decrease in vascular tone is compensated by an increase in

cardiac output, so that the blood pressure is maintained at the control level, or, more commonly, elevated slightly above the control level. In the second type of reaction there is no compensatory increase in cardiac output, so that the blood pressure falls as the vascular tone decreases. The sweating and hyperperistalsis which accompany the collapse reaction are signs of cholinergic stimulation and suggest that the cardiac rate is inhibited by strong vagal activity. Lewis (11) found that the administration of atropine prevented the fall in heart rate but not the drop in blood pressure.

In the present study there was a small group of subjects who demonstrated a third type of cardiovascular reaction to anxiety. In this group there was an overall increase in vascular tone. The blood pressure was maintained at or above the control level, while the cardiac output fell. The heart rate showed no material change or a moderate increase.

The extent of the fall in peripheral resistance which can occur in the anxious state is a phenomenon almost as striking as the increase in cardiac output. In contrast the mean blood pressure, which may be expressed as the product of the cardiac output and peripheral resistance, changes relatively little. The moderate elevation of blood pressure commonly seen in anxiety in normal persons usually represents a relatively slight imbalance between a large increase in cardiac output and a large fall in peripheral resistance. Starr (12) has described a group of persons with hyperactive circulation and a moderate elevation of blood pressure. He points out that in these persons the elevation of blood pressure is on the basis of an increased cardiac output, while in persons with essential hypertension the peripheral resistance is increased and the cardiac output is normal or even sub-normal. It is to be emphasized, however, that increases of blood pressure in normal persons during anxiety are of only moderate degree. Normally, the peripheral resistance and cardiac output are so adjusted during anxiety as to prevent any large change in mean arterial pressure. Failure to accomplish such an adjustment may, in itself, be indicative of arterial dysfunction.

There is considerable evidence that various patterns of vasoconstriction and vasodilatation may

be centrally set up in response to different stimuli to the nervous system. The Lovén reflex, in which stimulation of afferent fibers from an organ produces generalized vasoconstriction and localized vasodilatation, is an example of this phenomenon. Emotional blushing reactions of the skin and intestinal mucosa are further examples of a localized vasodilatation resulting from a central stimulus. On the other hand, pain produced by the immersion of an extremity in ice water may be associated with a large increase in peripheral resistance and an elevation in blood pressure without any apparent localized regions of vasodilatation. While it is true that the vasomotor pathways through the sympathetic chain normally exhibit a strong constrictor tone, it is apparent this tone can be increased or decreased in various anatomic patterns by the vasomotor centers in response to different stimuli.

There appears no need to postulate that emotional stimuli which may incite activity of the sympathetic nervous system need be associated with generalized vasoconstriction or with any fixed vascular pattern. It seems likely that further experience with anxious subjects will demonstrate additional types of cardiovascular reactions.

The nature and location of the vascular channels through which the excess quantity of blood circulates in those persons who develop a hyperactive circulation in response to anxiety is a problem of considerable interest. The disproportion of cardiac output to oxygen consumption and the high oxygen content of mixed venous blood suggest that a considerable portion of the output is shunted through arterio-venous connections or preferential channels thus by-passing the capillaries. There appears to be no present evidence to indicate the organs in which such shunts are occurring.

The extent of the extra cardiac work which may be imposed by anxiety is impressive. The average cardiac index of the student group (non-basal) was elevated from 4.2 in the control period to 6.2 in the anxious period. From previous studies of the normal response of the cardiac output to the oxygen requirements of the body, it is estimated that this increase in cardiac output corresponds very roughly to that which would be demanded by an increase in oxygen consump-

tion equal to the basal metabolism. The extra cardiac work imposed by anxiety may well be a contributory factor in the production of attacks of angina pectoris during physical rest in persons with impaired coronary circulation.

Previous studies have shown that patients with congestive heart failure at rest have an abnormal response to exercise. They have a small increase or an actual decrease in cardiac output and a marked rise in pulmonary arterial pressure. The rise in pulmonary arterial pressure seems to be related to the inability of the left ventricle normally to increase its output and probably in part represents a rise in left atrial and pulmonary capillary pressures. In view of the abnormal response to exercise, a stimulus normally causing a rise in cardiac output, it would be logical to expect an abnormal response to anxiety, a stimulus again normally causing a rise in cardiac output. In one patient with congestive failure, anxiety caused changes similar to but less marked than those produced by exercise. It is suggested that in certain patients with heart failure acute attacks of pulmonary edema may be precipitated by anxiety because of the disturbance in pulmonary pressures from the inability of the left ventricle to respond normally. This mechanism may be responsible for some of the attacks of acute pulmonary edema precipitated by procedures as thoracentesis, abdominal paracentesis, catheterization, or lumbar puncture.

SUMMARY AND CONCLUSIONS

1. In the majority of persons, anxiety has an effect on the circulation similar to that produced by small doses of epinephrine. The cardiac output, heart rate, and oxygen consumption are increased. There is a moderate elevation of blood pressure, but the peripheral resistance is decreased. The cardiac output is abnormally high in proportion to the rate of oxygen consumption. When persons with this reaction undertake muscular exercise, the normal relation between cardiac output and rate of oxygen consumption is re-established.

2. In certain subjects anxiety may be attended by circulatory collapse. In such cases the peripheral resistance is decreased, but the compensatory increase in cardiac output fails to counteract the resultant fall of blood pressure to a level

3. In a small group of subjects anxiety results in an increase in peripheral resistance and an elevation of blood pressure, with no change or a fall in cardiac output.

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LETTER FROM THE EDITORS

It may be of general interest to the contributors and subscribers to explain some of the vicissitudes of the editors, the problems of the mechanics of editing and printing the *Journal* and at times to introduce miscellaneous topics for attention and comment. This procedure reverses the customary letter to the editors in which they are targets for criticism or correction—enough comments still come in to remind us that we are not infallible.

Assuming that an article submitted is worthy of publication, it follows that the sooner it appears in print the better, not just for the credit of priority but for the spread of information in fields of investigation where the advance is rapid. The usual processing of manuscripts begins with their examination by each of the editors, discussion at a weekly meeting and except in rare instances, the selection of a minimum of two reviewers for careful criticism. This initial processing takes one or two weeks, and averages about ten days. Reviewers are asked to return the manuscripts within 14 days, and this has been adhered to with increasing frequency; the average now is two weeks while formerly it was slightly more than four weeks. The submission of two copies of a manuscript has reduced the time spent by editors and reviewers by nearly half, so that final action may be taken on a paper within three weeks whereas formerly the minimum was five or six weeks.

In a representative issue approximately one-third of the accepted articles do not require alteration or revision. The remainder may require slight or extensive change, and on the average this has taken just over five weeks with extremes of one to 15 weeks.

Copy is sent to the printer ten weeks before the first day of the month of issue. While it may be possible to shorten this period of typesetting, return and correction of galley proofs and page proofs, and finally printing and distributing the *Journal*, this has not been done so far. Assuming that a manuscript went through the stages in the shortest possible time it might appear in the *Journal* in just over three months and we have had a few appear less than four months after they were received. The average time elapsing for articles in the last issue was just under six months. This is approximately the same time as for two random numbers in 1940, a sample pre-war year, and it compares with a period of about ten months in the war year 1944, when the *Journal* was actually mailed much later than the month noted on it.

This brief survey may clarify to those not familiar with the problem some of the reasons why papers do not get printed more rapidly. We are actually approaching very favorable elapsed time performance in handling manuscripts, and for this we must give credit to our reviewers as well as the contributors. We hope for even more improvement, but there is a minimum beyond which we cannot go.

EFFECTS OF HYPOXIA AND HYPERCAPNIA ON PERCEPTION OF THERMAL CUTANEOUS PAIN

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Asphyxia, which combines depletion of oxygen (hypoxia) with accumulation of carbon dioxide (hypercapnia), is known to modify sensation. While prolonged, mild hypoxia impairs vision and certain other sensations (1), its effect on the perception of thermal pain has not been described. Similarly, severe hypercapnia produced by breathing 30 per cent carbon dioxide produces anesthesia (2, 3), but the analgetic effects of lower concentrations have received only brief consideration (4, 5). The present studies were undertaken to determine whether hypoxia or hypercapnia is responsible for the analgesia of asphyxia. The results led secondarily to a comparison of carbon dioxide and nitrous oxide with respect to their quantitative effects on pain perception and their site of action.

SUBJECTS AND METHOD

Observations were made on 14 healthy male subjects between the ages of 20 and 45 years. Eleven were medical students, and three were members of the teaching staff.

Apparatus and methods were quite similar to those described by Hardy, Wolff, and Goodell (6) except for two minor modifications of technique. Stimuli, consisting of graded radiant heat, from a 1000-watt bulb and lens, previously calibrated by a radiometer, were applied for exactly three seconds to 3.5 square centimeters of blackened skin on the forehead and in some experiments on the forearm. "Threshold for pain" was taken as the lowest rate of heating, expressed in gm. cal. per cm.² per sec., which produced a clear, "sticking" pain, i.e., a sensation of sharp, needle-like penetration into the skin, as described by Chapman and Jones (7).

Two slight modifications were introduced (a) because of the rapid changes in blood gases produced by modifying the composition of inspired air, and (b) because it was necessary to measure changes in threshold quickly to avoid unduly prolonged exposure to abnormal gas mixtures. Stimuli were, therefore, applied for three seconds out of each 30 seconds instead of each one or two minutes as advised when more slowly developing changes are followed, e.g., effects of drugs. For reasons described below it was found very important under these conditions to

prevent local ischemia. Therefore, the exposed skin rested against a radially ridged celluloid plate, rather than immediately against the edges of the aperture itself.

Gas mixtures were prepared in a bank of Tissot recording spirometers (capacity of each 100 liters) from which the subject breathed out to the atmosphere. A three-way valve permitted shifting from one mixture to another without the knowledge of the subject. The mouthpiece and nose-clip were carefully adjusted to avoid discomfort. Arterial blood pressure, heart rate, respiratory minute volume and respiratory rate were recorded during the observations on hypoxia and hypercapnia.

OBSERVATIONS

1. The effect of local ischemia and its avoidance

The necessity in these observations of determining changes in threshold rapidly has been mentioned. As illustrated in Figure 1 (left) stimuli were applied every 30 seconds and reported by the subject as "clear sticking pain" (solid dots), "doubtful" (half shaded circles) and "not painful" (open circles). The intensity of stimulus was changed frequently so that the accuracy of each threshold stimulus was verified by one or more sub-threshold stimuli in close proximity. In Figure 1 (left) all the readings taken are included to show general procedure, but in other charts only the threshold values themselves are given. In this manner five or more verified determinations of threshold could usually be obtained in 7½ minutes as shown by the solid dots connected by solid lines.

Control observations with such stimulation every 30 seconds yielded results which were at first considerably more variable than those described for stimulation every one to two minutes. This variability appeared to be related in part to the degree of firmness with which the subject pressed his forehead against the edges of the aperture. As shown in Figure 1 (left) the threshold observed when the forehead was resting lightly against the head rest was measurably higher than when the subject purposely held his forehead in place with

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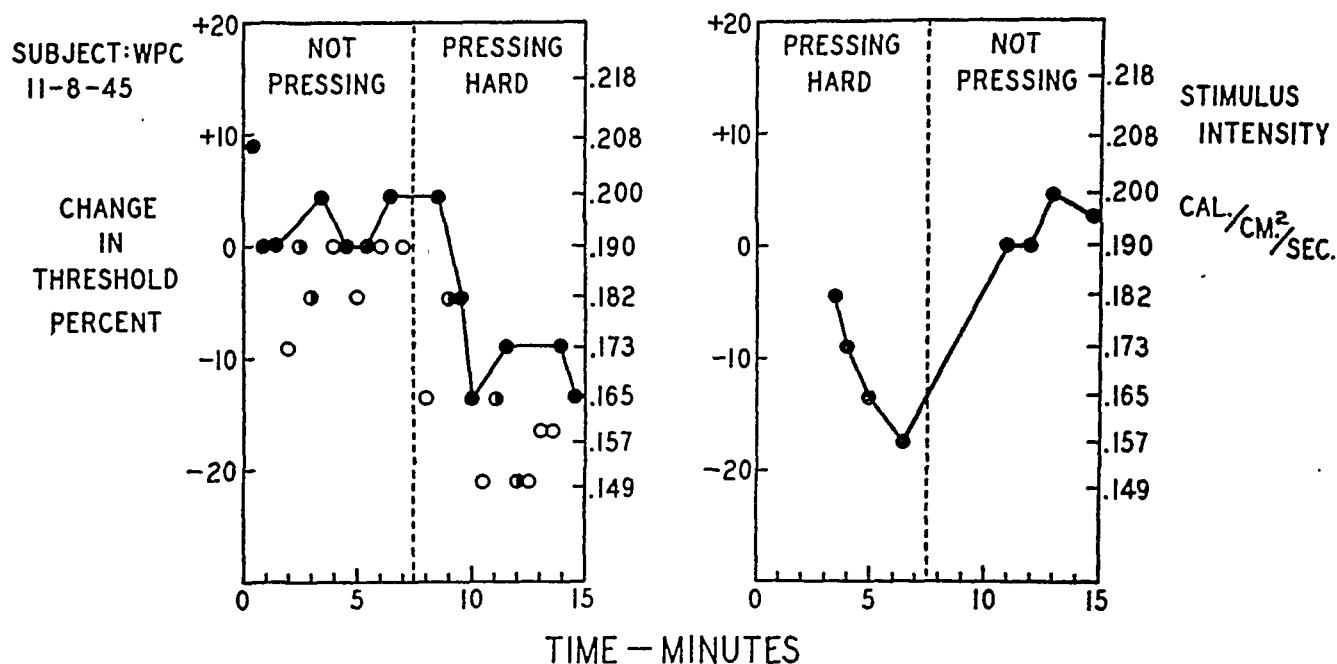


FIG. 1. CHART SHOWING EFFECT ON THRESHOLD OF PRESSING FOREHEAD FIRMLY AGAINST THE APERTURE TO PRODUCE LOCAL ISCHEMIA

To the left all responses are charted to show subthreshold (open circles and half open circles) and threshold responses (dots). To the right, as in all subsequent charts, only threshold responses are charted.

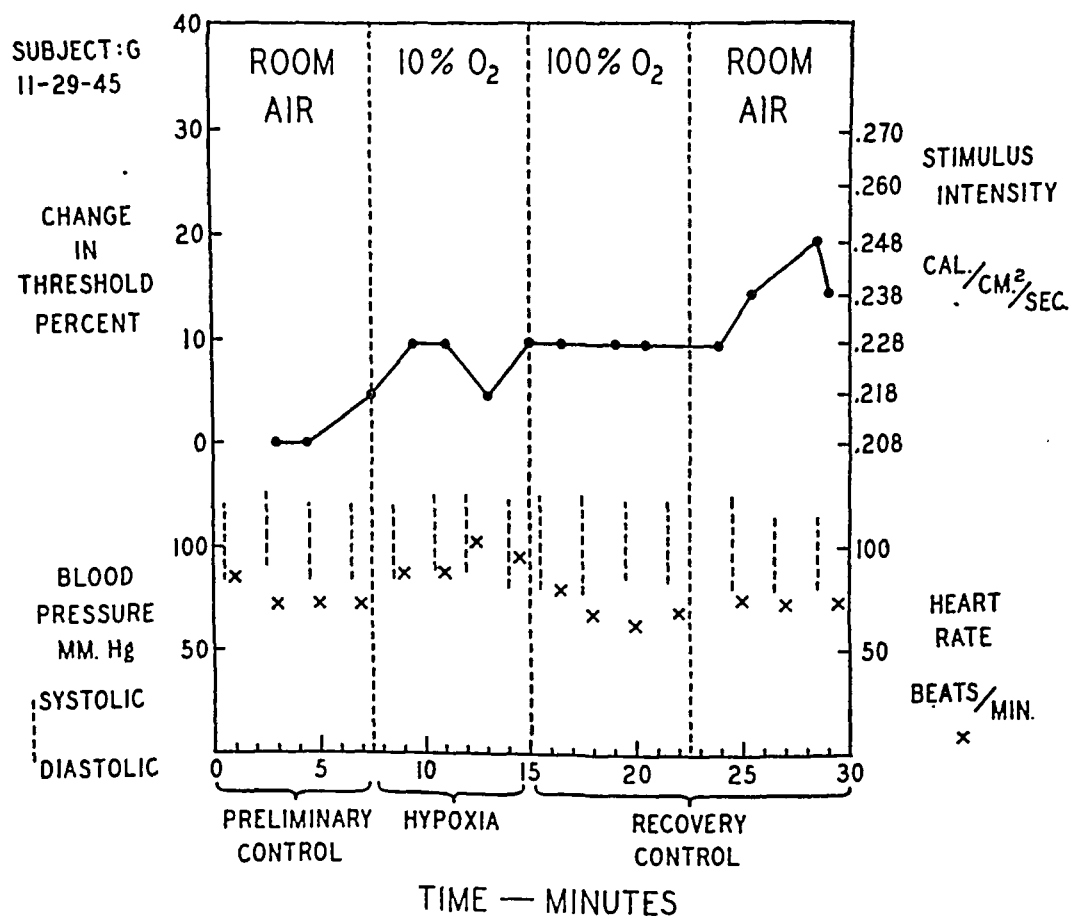


FIG. 2. CHART SHOWING ABSENCE OF SIGNIFICANT CHANGE IN PAIN THRESHOLD DURING ACUTE ANOXIA

firm, but not painful, pressure. An observation in reverse order is shown to the right of Figure 1.

It is a common observation that water at 44.5° C. is usually not painful to the extremities when circulation is normal but may become intolerably painful when circulation is arrested and convection of heat by blood thereby eliminated. Therefore it seemed likely that pressing the skin of the forehead against the edge of the aperture might, particularly in the mid-line, produce local ischemia and thereby change threshold. In addition, stimulation at intervals of 30 seconds afforded less opportunity for interim cooling by radiation and air convection than is provided by conventional stimulation every one or two minutes.

Lewis and Pochin (8) showed that prolonged local asphyxia is required to modify several types of cutaneous pain sensation, the late changes being a diminution and loss in pain sense. With heat, however, Bigelow *et al.* (9; see Figure 3, p. 507) observed in the forearm first a fall in threshold for both burning pain and pricking pain followed after 10 to 20 minutes by analgesia. Likewise, in our experiments local interference with circulation was associated with a reduction of pain threshold (Figure 1). This observation was verified by other studies on the forearm (Figure 4) in which painless arrest of blood flow with a pneumatic cuff on the upper arm reduced threshold by 20 per cent. In repeated thermal stimulation of one skin area the effect of local ischemia on pain threshold must be due in part to reduced dissipation of heat by the circulation, but the simultaneous effect of local asphyxia of the stimulated nerve endings may also play a role (9).

Whenever the forehead was used, errors from this "ischemia artefact" were avoided by placing around the aperture a circle of celluloid with six radiating spokes 2 mm. high. Pressing the forehead firmly, but painlessly, against these supports did not affect threshold presumably because blood continued to flow through blood vessels between the spokes, and interim cooling was more effective. Even when this artefact was excluded thresholds were still not entirely constant because most subjects showed some adaptation to repeated thermal stimulation, both acutely and chronically. In each series of 60 exposures (compare initial and terminal control periods in Figures 2, 3 and 4)

threshold rose slowly and by varying amounts. Repeated experiments once or twice weekly on the same subjects over a period of five weeks also revealed a slow rise of threshold averaging less than 15 per cent. This slow adaptation did not affect perceptibly the acute changes produced by experimental procedures. It could not be eliminated but it was controlled by keeping the total number of exposures constant for each observation.

2. The effects of hypoxia

Figure 2 illustrates a typical experiment in which the subject breathed (a) room air for a control period of 7.5 minutes, (b) 10 per cent oxygen in nitrogen for 7.5 minutes, (c) 100 per cent oxygen for a like period and finally room air again for a terminal control period. The results in a total of six subjects are summarized in Table I.

TABLE I

Comparison of the effects produced by hypoxia, hypercapnia, voluntary hyperventilation and nitrous oxide

Procedure	Number of subjects	Average percentage increase of			
		Heart rate	Blood pressure, syst./diast.	Minute volume	Pain threshold
Hypoxia					
10% O ₂ in N ₂	6	26	0/0	37	0
Hypercapnia					
5% CO ₂ in O ₂	9	6	15/9	280	13
7.5% CO ₂ in O ₂	9	13	23/21	380	28
Voluntary hyperventilation					
Room air	2	12	4/0	310	4
30% N ₂ O in O ₂	4				27

Anoxia sufficient to increase heart rate by an average of 26 per cent and ventilation by an average of 37 per cent had no significant effect on threshold for thermal pain.

Figure 2 shows also the apparent fluctuations in threshold which demonstrate the subject's inability to differentiate between changes of stimulus intensity amounting to less than 5 per cent. The gradual rise in threshold is characteristic of adaptation and not related to the period of hypoxia. It appears, therefore, that hypoxia affects pain sensation far less than certain other sensations, e.g., vision (10).

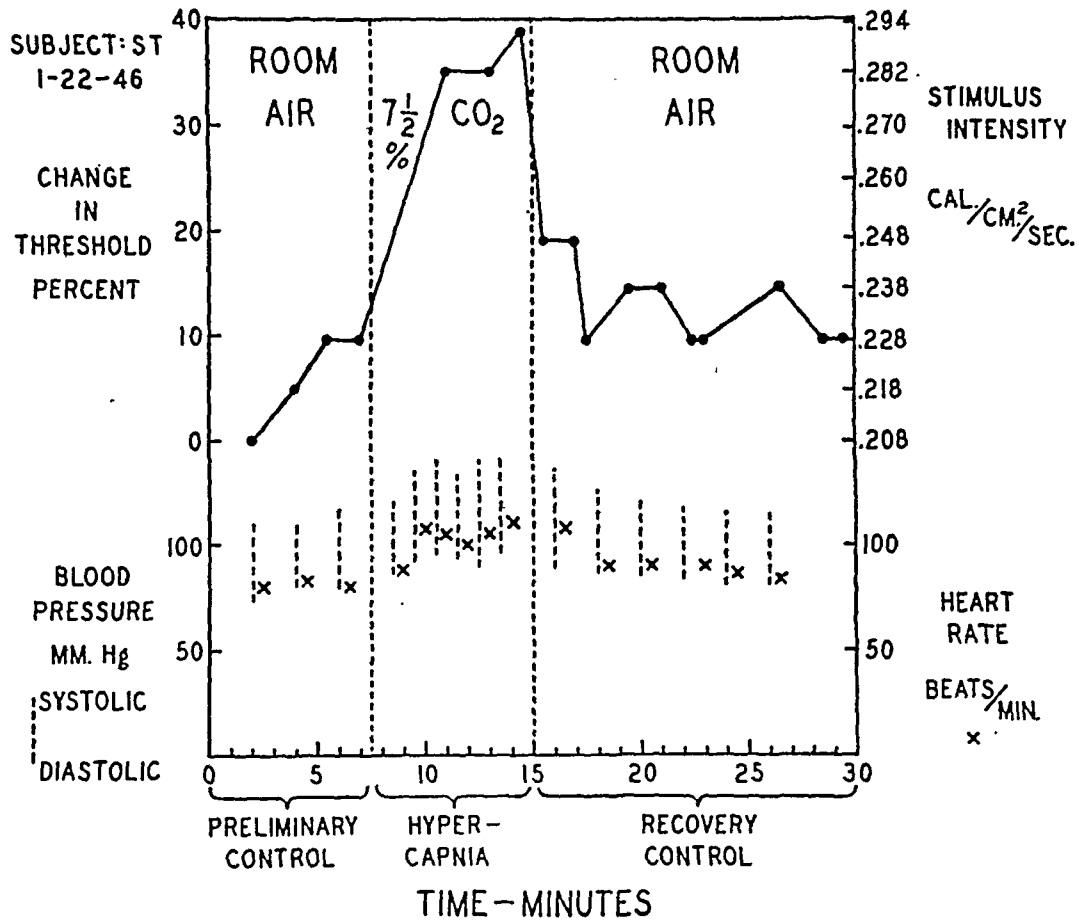


FIG. 3. CHART SHOWING THE EFFECT ON PAIN THRESHOLD OF BREATHING 7.5 PER CENT CARBON DIOXIDE FOR 7.5 MINUTES

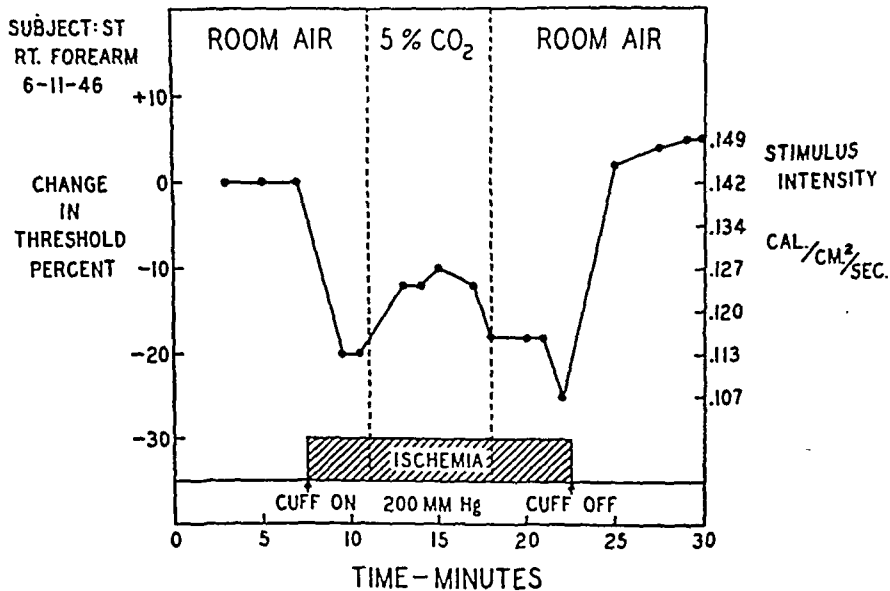


FIG. 4. CHART SHOWING (a) THE DECREASE IN THRESHOLD IN THE FOREARM PRODUCED BY ARRESTING BLOOD FLOW AND (b) THE RISE OF THRESHOLD PRODUCED DURING THIS ISCHEMIA BY INHALING CARBON DIOXIDE

This indicates that carbon dioxide produces analgesia by central action rather than by direct effect on peripheral pain receptors.

3. *The effects of hypercapnia*

In striking contrast to hypoxia, moderate hypercapnia produced by breathing mixtures of 5 and 7.5 per cent CO_2 elevated threshold significantly in all subjects. The response of subject ST to 7.5 per cent CO_2 is shown in Figure 3 and the data on nine subjects are averaged in Table I. In each subject threshold rose abruptly by at least 9 per cent while 5 per cent CO_2 was breathed and by at least 18 per cent while 7.5 per cent CO_2 was breathed. The average effects were a 13 per cent and a 28 per cent rise in threshold, respectively. Simultaneously blood pressure and heart rate increased moderately while minute volume rose markedly.

Before concluding that the rise in threshold was due solely to a direct analgetic effect of CO_2 it was necessary to exclude the possibility that other physiological responses to hypercapnia were involved. (a) The mild to moderate sweating, which hypercapnia produces, can be excluded because it has been shown that profuse sweating does not influence the threshold for thermal pain (7). (b) Though breathing carbon dioxide produces vasodilatation in the finger tips (11) blood flow to the skin of the forehead is not likely to be affected by inhalation of carbon dioxide because flow is always rapid and not subject to vasoconstrictor influences. The breathing of carbon dioxide did not change skin temperature of the forehead measured by thermocouple in experiments similar to those in Figure 3 except for omitting the intermittent application of radiant heat. (c) Though several subjects mentioned the distraction and mild discomfort experienced during the period of hyperpnea, voluntary hyperventilation to match the hyperpnea produced by CO_2 did not affect threshold significantly (Table I). It may be concluded that carbon dioxide can produce specific analgesia and that in early asphyxia pain sensation is affected more by the accumulation of carbon dioxide than by depletion of oxygen.

4. *The central vs. the peripheral action of carbon dioxide*

Carbon dioxide might conceivably produce its effects either by acting upon sensory end organs in the skin, or upon more centrally located parts of the nervous system. In order to distinguish be-

tween these two possibilities an area of skin on the flexor surface of the forearm was blackened and exposed to heat stimuli (a) with normal circulation, (b) during total occlusion of blood flow by inflating a pneumatic cuff on the upper arm to 250 mm. Hg and then (c) with added inhalation of 5 per cent CO_2 . This procedure permitted the inhaled CO_2 to reach the central nervous system but not the end organs in the ischemic forearm. It has already been mentioned that very prolonged ischemia is necessary to change pain sensation by direct action on peripheral receptors (8).

Figure 4 illustrates the type of response observed in three subjects. Superimposed upon the fall in threshold to be expected from arrest of blood flow (see also Figure 1), the inhalation of 5 per cent CO_2 produced the usual rise of threshold. Shifting the subject to room air permitted threshold to fall again to the level characteristic of ischemia alone and restoring blood flow elevated the threshold to normal. This indicates that the analgetic effect of carbon dioxide is not peripheral, but chiefly central.

DISCUSSION

It is interesting to compare the side effects of carbon dioxide with those of intermediate concentrations of nitrous oxide which are known to be definitely analgetic (12). In three subjects 30 per cent nitrous oxide elevated threshold by an average of 27 per cent in agreement with previous work (12). While breathing this concentration of nitrous oxide subjects described euphoria, dissociation with their surroundings and ringing in the ears. Inhalation of 7.5 per cent CO_2 elevated threshold by a similar amount, 28 per cent, but the sensations were otherwise limited to the increase in respiratory movement with transient headache in some instances during or immediately following the experiment. Additional studies, similar to those illustrated in Figure 4, showed that nitrous oxide, like CO_2 , produces analgesia by central, rather than peripheral, action.

SUMMARY

Threshold for thermal pain was determined in healthy male subjects during moderate hypoxia and hypercapnia. An "artifact" form of ischemia is described. This consists of a lowering of threshold which is produced when arrest of circulation

interferes with the convection of heat away from the stimulated area by blood flow and with blood supply to the pain endings in the area being tested. It is particularly important when stimuli are repeated at short intervals.

Breathing 10 per cent oxygen did not affect pain threshold significantly while 5 and 7.5 per cent carbon dioxide elevated threshold by an average of 13 to 28 per cent, respectively. These effects were found to be due to central action rather than to any peripheral effect on the pain end organs. Nitrous oxide in 30 per cent concentration affected pain threshold similarly and also acted centrally.

The analgesia of *early* asphyxia appears to be due to the central action of accumulated carbon dioxide, not to lowering of oxygen tension, though the latter in sufficient grade is known to produce disorientation and sudden unconsciousness eventually.

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THE USE OF CONCENTRATED HUMAN SERUM ALBUMIN IN THE TREATMENT OF CIRRHOSIS OF THE LIVER¹

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The formation of ascites in patients with cirrhosis of the liver probably depends on at least three factors: (1) hypoalbuminemia, (2) portal hypertension, and (3) increased amounts of circulating antidiuretic substance. The relative importance of these factors varies with the individual patients. Although the formation and disappearance of ascites do not correlate perfectly with levels of serum albumin in all patients, in the majority of cases the two findings are coincident and apparently related (1, 2). The use of concentrated human serum albumin in these patients for its osmotic effect would be expected to cause a diuresis. The extent to which this diuresis is produced would depend in large part upon the relative importance of the various factors of ascites production, being most marked in those patients in whom hypoalbuminemia is most specifically at fault.

The observations of Janeway (3) and Thorn (4) and their co-workers in patients with liver disease demonstrated that the albumin level of the serum could be raised to normal by repeated injections of albumin. Certain of their patients showed a loss of edema and ascites but the oncotic effects were disappointing in their experience.

¹ The serum albumin used in this study was supplied in part by the Bureau of Medicine and Surgery, United States Navy, and in part by the American National Red Cross. It was prepared from blood collected by the American Red Cross from voluntary donors.

This is one of a series of investigations on serum albumin being carried out with material supplied by the American National Red Cross. As soon as sufficient data become available to justify final conclusions concerning its therapeutic value, a full report to the medical profession on the use of serum albumin in medical practice will be published.

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While the largest portion of the administered albumin did not remain in the blood stream, nitrogen balance studies showed that this protein had been retained, presumably in the tissues. The question as to whether parenterally administered albumin is utilized in building tissue protein is not completely clear, however. The work of Whipple and co-workers (5, 6) in dogs has indicated that plasma protein may replace tissue protein without being broken down to its constituent amino acids. Albright and co-workers (7) by measuring phosphorus and potassium as well as nitrogen balance, have obtained evidence for the utilization of part of the injected albumin for the building of tissue protein in certain patients.

In addition to its oncotic and nutritional properties, albumin probably has other important functions in the body. It has the remarkable capacity to enter into combination with many molecules and ions in a manner different from other proteins (8). This property also has made albumin a valuable supplement to a medium for the growth of tubercle bacilli (9). The significance of the binding effect of albumin in the living organism has not been fully determined but it undoubtedly is important in the transport of various substances through the body. In view of these specific effects of albumin, it is to be expected that albumin therapy in liver disease would produce profound metabolic results.

MATERIALS AND METHODS

The present study represents the results of albumin therapy in 17 patients with severe liver disease. All but two of them demonstrated ascites at the time when albumin was first administered. The diagnosis was well-established by a characteristic history, numerous physical examinations, x-ray studies of the thorax and abdomen, and a wide variety of liver function studies. In eight of the patients the diagnosis was confirmed by the gross appearance of the liver and from sections of the liver obtained at laparotomy or autopsy. All patients were hospitalized at the Hospital of The Rockefeller

TABLE I
Summary of clinical and laboratory data before and after albumin therapy in 17 cases of chronic liver disease

Case no.	Age	Sex	Clinical data before albumin				Immediate response to therapy	Duration of follow-up after therapy	Final outcome of therapy				
			Albumin retention	Bilirubin	Intestinal treatment	Initial treatment			$\frac{A}{G}$	Albumin retention	Bilirubin		
GROUP I													
1	42	M	Alcoholic. Large liver, large spleen. Following 3rd esophageal hemorrhage developed ascites that did not respond to 3 mos. of dietary therapy. General condition was fair. 4 paracenteses.	2.8 3.6	15	0.6	16 units in 4 days	Immediate diuresis with loss of all ascites. Improvement in appetite and general condition.	12 mos.	3 subsequent small hemorrhages following return to alcohol. Slight ascites after 3rd hemorrhage which disappeared on dietary therapy over a period of 2 mos.	2.5 3.3	11	0.5
2	45	F	Nutritional deficiency. Gradual development of edema and ascites despite mercupurin. Small liver, large spleen. 1 paracentesis. 1 hemorrhage. Ascites for 3 mos.	2.6 3.6	19	0.8	8 units in 2 days	Immediate diuresis with complete loss of edema and ascites. Small esophageal hemorrhage at height of diuresis and 2 transfusions given.	9 mos.	Continued improvement in appetite and general condition. Returned to full activity. No recurrence of ascites. Marked gain in body weight.	2.5 5.5	12	0.7
3	32	F	Jaundice and esophageal hemorrhages over a period of 9 yrs. Etiology of cirrhosis obscure. Moderate edema and ascites. Responded to i.v. liver extract therapy for 3 mos. but patient again developed marked fluid on this therapy. Large liver, large spleen. Ascites for 1 month.	2.4 3.0	37	3.4	21 units in 1 mo.	Initial diuresis with loss of edema fluid. Ascites persisted. Sudden loss of ascites 2 wks. after albumin was stopped. Increased appetite and strength.	16 mos.	No further ascites. Edema appeared during menstrual periods. Appetite remained good. Returned to full activity. Serum albumin gradually decreased.	1.0 2.3	34	1.2
4	47	M	Jaundice and abdominal pain at age of 45. Intermittent jaundice persisted. 3 esophageal hemorrhages followed by generalized edema, ascites and chest fluid. Large liver and spleen. Albumin given because of respiratory difficulty. Ascites for 1 month.	2.2 3.6	37	12	10 units in 6 days	Immediate respiratory relief. Gradual loss of edema and ascites over period of 2 wks. Considerable general improvement.	2 mos.	Remained improved for 2 wks. and then developed another hemorrhage with coma for 4 days. Gradual recovery. Died following a spleno-renal anastomosis.	1.2 3.6		
5	18	F	Infectious hepatitis at age of 13. Intermittent jaundice subsequently. Esophageal hemorrhages at 17. After 1st hemorrhage edema and ascites developed requiring 2 paracenteses. This cleared in 4 mos. on dietary therapy. Small liver, large spleen. Ascites returned after 3rd hemorrhage.	2.1 3.0	40	1.2	10 units in 3 days	Immediate and marked diuresis with complete loss of ascites. Albumin was rapidly brought to normal and remained normal until after spleno-renal anastomosis.	10 mos.	Normal serum albumin was sustained and no further ascites appeared for 3 mos. A 4th hemorrhage resulted in ascites which responded to 10 additional units of albumin. A 5th hemorrhage occurred during this therapy but ascites continued to disappear. Spleno-renal anastomosis performed to control hematemesis. Edema and ascites following operation disappeared after 15 additional units of albumin. Liver showed post necrotic cirrhosis.	1.5 2.9	50	1.8
GROUP II													
6	47	M	Jaundice at age of 16. Alcoholic. Marked weight loss. Ascites and edema for 8 mos. 30 paracenteses. Enlarged liver. 6 mos. trial on liver extract, choline, high protein diet and vitamins, but weight loss continued. 1 esophageal hemorrhage. Terminal state when albumin was begun.	2.8 4.1	30	2.6	20 units in 3 mos.	Very little effect at first - a gradual increase in urine output and decrease in rate of fluid accumulation. Doses of 4 units per day were necessary to raise serum albumin to normal. A delayed diuresis occurred 3 wks. after albumin was stopped. Dietary intake rose from 2000 to 4000 calories.	15 mos.	Almost complete recovery. Patient returned to full time work. Reaccumulation of fluid 5 mos. after first course of therapy. 15 additional units caused a slow disappearance of this fluid. No further paracenteses. Marked gain in body weight. Regained ability to synthesize albumin normally 1 yr. after albumin was started.	4.1 3.2	11	0.8
7	36	F	Alcoholic. Ascites for 1 yr. 4 paracenteses. Mercupurin injections regularly. 6 mos. on choline without effect. Large liver and spleen. Some loss of edema following hospitalization for 6 wks. Fluid was accumulating again when albumin was begun.	2.7 4.5	39	4.5	24 units in 6 wks.	Very gradual loss of both edema and ascites despite normal serum albumin levels for more than 6 wks. Mercupurin more effective following albumin. Markedly improved appetite and general condition.	7 mos.	Complete loss of edema and ascites. Gain in body weight. Return to normal activity. Slight ascites after 6 mos. but remained in good general condition.	1.6 3.5	28	1.7
8	68	F	Infectious hepatitis at age of 25 and again at 45. Liver not enlarged. 8 paracenteses during 5 mos. Prior to albumin therapy. Liver extract for 2 mos. Body weight loss continued. Red-riden.	2.1 2.8	30	1.1	27 units in 1 mo.	After 2 wks. of therapy gradual loss of edema and most of ascites. Marked improvement in appetite and general condition.	9 mos.	Returned to normal activity. Ascites returned after 2 mos. and paracenteses were again resorted to, but these were less frequent and patient's general condition remained good. Died suddenly because of intestinal obstruction unrelated to the liver disease. Autopsy showed Laennec's cirrhosis.	2.3 3.0	31	0.8
9	54	F	Etiology unknown. Insidious development of ascites; present for 9 mos. 23 paracenteses. Extracted ascites as much as 20 liters removed at one time. No effect from 5 wks. i.v. liver extract. Small liver, large spleen.	2.7 2.6	16	0.7	20 units in 3 mos.	No diuretic response despite an approximate normal serum albumin level for 2 mos. No change in appetite or dietary intake.	6 wks.	No definite response to therapy. Paracenteses continue to be necessary every 2 wks.	2.7 2.5	20	0.6

TABLE I—Continued

Case no.	Age	Sex	Clinical data before albumin				Initial treatment	Immediate response to therapy	Duration of follow-up after therapy	Final outcome of therapy			
			Broth-albumin retention	A	%	Age				Broth-albumin retention	A	%	
10727 III													
10	32	M	Infectious hepatitis 18 mos. previously. Marked jaundice. 6 mos. course of liver extract, choline, high protein diet and vitamins had no effect. Tried to work but was unable to do so. Marked weight loss. Terminal anorexia when albumin was begun. Very large liver, spider angiomas, bleeding gums, large spleen. Ascites for 4 mos.	1.1 4.7	30	12	27	Marked immediate diuretic effect with complete loss of edema and ascites. Feeling of well-being and improvement in appetite. Returned to general activity for 3 mos. Bleeding gums persisted. Prothrombin time 30 sec.	11 mos.	No further paracenteses. Edema and ascites returned repeatedly and were easily controlled by additional albumin. 60 additional units were administered during 11 mos. Serum albumin and fluid balance were well controlled. Condition improved because of uncontrolled bleeding from gums and epistaxis. Prothrombin time 30 sec. Prothrombin time 34 sec. Fibrinogen 130 mg.%. Autopsy showed post necrotic cirrhosis.	1.0 3.7	31	25
11	37	M	Gradual onset of jaundice, edema and ascites at age of 10. Bilirubin 10 mg.%. 1 paracentesis. Marked weight loss. Large liver and spleen. Ascites for 2 mos.	1.9 3.7	31	4.2	4	Immediate diuresis with loss of edema and ascites. General improvement with increase in appetite and strength.	11 mos.	Hydrothorax without ascites appeared approximately 2 mos. later with immediate response to a smaller dose of albumin. On 5 occasions albumin was administered with a rapid and complete diuresis each time and associated with marked improvement in the progressive of Wilson's disease. Liver showed post necrotic cirrhosis.	1.0 1.9	27	5.6
12	31	F	Severe diarrhea and jaundice at age of 49. Jaundice persisted. Edema and ascites developed 1 yr. later. Controlled for 8 mos. by i.v. liver extract and dietary therapy. Gradual decline and in terminal state when albumin was begun. Ascites for 3 mos.	1.9 2.7	30	11	24	Immediate diuresis with complete loss of edema and ascites. Slight general improvement.	2 mos.	Jaundice increased following therapy. Ascites reappeared. Died in cholelasis. Autopsy showed biliary cirrhosis.	2.6 2.5	34	20
13	35	F	Jaundice, fever and arthritis for 2 yrs. Marked edema and ascites for 3 mos. 2 mos. later, liver extract administered with albumin because of terminal state. Large liver and spleen.	1.6 0.5	31	3.0	25	Marked diuresis after 5 units of albumin. Loss of all edema and ascites. Improvement in appetite and general condition.	28 mos.	Free of symptoms of liver disease for 2 yrs. despite persistent abnormalities of liver function. Regained ability to form albumin for 2 yrs. Laparotomy biopsy revealed that definite cirrhosis persisted. i.v. liver extract administered throughout course. Regained ascites 4 mos. ago which responded to 10 additional units of albumin.	1.0 1.4	16	0.8
14	36	F	Infectious hepatitis at age of 36. Complete recovery. Relieved 3 mos. before therapy followed by liver extract and albumin. 1 paracentesis. Large liver and spleen. Constant condition when albumin was begun.	1.4 3.2	32	8	8	Diuresis with considerable loss of edema and ascites while in comatose state. No general improvement.	4 days	Patient died in coma. Albumin did not alter course despite diuresis. Liver showed subacute yellow atrophy.	1.1 2.9	15	15
15	10	M	Infectious hepatitis with transition to edema and ascites 6 wks. later. Anasarca and terminal state when albumin was begun.	2.0 1.8	16	6	6	Diuresis with loss of edema and a large amount of ascites. No change in general condition.	10 days	Died despite diuresis. Downhill course of illness not affected by albumin. Liver showed subacute yellow atrophy.	1.1 2.7	21	21
16	16	M	Infectious hepatitis at age of 29. Recovered. Infectious jaundice associated with bilateral Mott's disease 3 mos. before therapy. Large liver and spleen. No edema or ascites. No bleeding tendency.	2.2 4.9	22	6.6	6	Definite response in respect to appetite and general strength. Dietary intake increased.	6 mos.	Therapy raised serum albumin level and patient was able to maintain this by high caloric intake.	1.1 6.0	15	1.7
17	14	M	Infectious hepatitis 18 mos. before therapy. Persistent symptoms. Large liver and spleen. Numerous spider angiomas. No edema or ascites.	2.0 5.7	27	1.4	6	No definite response to albumin. Dietary intake remained unchanged.	7 mos.	Serum albumin level remained higher than prior to therapy. Laparotomy biopsy showed post necrotic cirrhosis.	1.4 5.0	24	1.6

Institute during the period of albumin therapy. When sufficient improvement had been accomplished by hospitalization, the patients were observed twice weekly in the Out Patient Department.

Etiology of the 15 cases of cirrhosis in this series was varied. Four gave a clear-cut history of chronic alcoholism with associated nutritional deficiencies; four were patients with cirrhosis of the liver following infectious hepatitis; in the remaining seven the etiology of the cirrhosis was obscure.

In addition, two patients with subacute yellow atrophy following infectious hepatitis were treated with albumin. Both of these patients had edema and ascites and were in a critical state when treatment was begun; the diagnosis was confirmed at autopsy.

In studies of the changes in caloric intake during the course of albumin therapy, careful records of the daily intake of protein, fat and carbohydrate were kept by trained dietitians. An effort was made to provide patients with an excess of food and calculations of the intake were made from the amounts offered and refused. Diets were kept high in protein; fats and carbohydrates were provided according to the taste of the individual. For nitrogen balance studies three-day collections of urine and feces were analyzed for total nitrogen by the macromethod of Kjeldahl; nitrogen intake was calculated from standard tables.

Salt was not restricted in the diet. Preliminary studies indicated that with such a restriction the caloric intake fell, and, since therapy was chiefly directed toward increasing caloric intake, salt restriction was not continued.

Alterations in serum proteins under the influence of albumin were studied in detail in each case. Serum albumin and globulin were determined by the Howe method (10) with Kjeldahl digestion and nesslerization of the filtrate. Electrophoretic analyses were carried out on the serum and ascitic fluid in certain of the patients as a supplementary method of protein estimation. The effect on the serum proteins of changes in plasma volume associated with albumin therapy was carefully observed; plasma volume determinations were carried out by the method of Gibson and Evans (11).

In determining changes in liver function during the course of therapy the methods employed were identical with those presented in a previous study (12). Estimation of serum esterase activity was carried out in the Warburg manometric apparatus and the results expressed in terms of the amount of CO_2 released from bicarbonate buffer following the hydrolysis of acetyl choline (13). The normal range is 40–80 mm. CO_2 .

The antidiuretic activity of urines of six patients was measured by a bio-assay technic (14) in which male rats were hydrated and then injected intraperitoneally with a standard amount of dialyzed and concentrated human urine. The minutes required for the excretion of 50 per cent of the ingested water was taken as the assay time, which in normals ranges from 80–140 minutes.

Method of albumin administration. Albumin^{*} was administered intravenously from a standard unit containing 25 gms. in 100 cc. of buffered diluent to which no preservative is added. This is the amount of albumin obtained from 500 cc. of plasma. The albumin used in 1945 contained 0.6–0.9 gms. of sodium per 100 cc., whereas in 1946 and 1947 the "salt-poor" product (0.3 gms.) was given. No marked difference between these two types in respect to diuretic effect could be ascertained.

TOXIC REACTION TO ALBUMIN

Four of the 17 patients treated with albumin developed a febrile reaction on one or more occasions approximately four hours after administration. None of these reactions was considered serious although a temperature of 104° was encountered once.

Two patients (Nos. 2 and 5) developed esophageal hemorrhages during the period of albumin therapy. This occurred at a time when marked shifts in body fluids were taking place and diuresis was at its height. In neither case was the hemorrhage serious. Since patient No. 5 had bled previously on numerous occasions, it is possible that the hemorrhage was coincidental.

Cardiac complications were not encountered in any of the patients. However, another patient, not included in this series, who was given albumin for other purposes, developed pulmonary edema following 14 units of albumin from which she recovered with great difficulty.

CLINICAL RESULTS

In order to evaluate the results of albumin therapy, the 17 patients in this series have been divided into four main groups: I. Patients treated soon after the onset of ascites; II. Patients with longstanding ascites who required frequent paracenteses; III. Patients suffering from a marked albumin deficit associated with unusually severe liver damage; and IV. Patients with low serum albumin levels without edema or ascites. Table I summarizes the results of therapy in the entire series.

Group I. Five patients comprise the group of cases with early ascites. Two were the nutritional type of cirrhosis, two were cirrhosis of undeter-

^{*} This material was prepared at the Harvard Fractionation Laboratories, Department of Physical Chemistry, from blood collected by the American Red Cross.

mined etiology, and one was cirrhosis after infectious hepatitis. Relatively small amounts of albumin produced a diuresis with loss of ascites and general improvement in each of the patients. Figure 1 illustrates the rapid loss of ascites in patient No. 5 in whom 10 units of albumin were sufficient to produce the desired diuretic effect.

Figure 2 illustrates the course of patient No. 3 who while on intravenous liver extract therapy showed some increase in the serum albumin level and some loss of the moderate edema and ascites that were present. However, the serum albumin subsequently fell and marked ascites developed. Following the administration of 9 units of albumin a diuresis occurred with loss of all edema and some ascites. Despite continued albumin therapy, further ascitic fluid accumulated and second para-

centesis was necessary. After 21 units were administered, albumin administration was discontinued. Approximately two weeks later a second diuresis occurred. This patient has been followed for more than one year after the loss of ascites and has remained well. Minimal edema still develops during menstrual periods but ascites has never reappeared. The serum albumin level gradually declined following the period of therapy so that it may be assumed that a defect in the synthesis of this protein still exists but is less severe. The patient gained approximately 15 lbs. of body weight and returned to normal activity. This case illustrates the marked improvement that may occur despite persistent severe damage and impaired ability to synthesize normal amounts of albumin.

The remaining three patients of the group im-

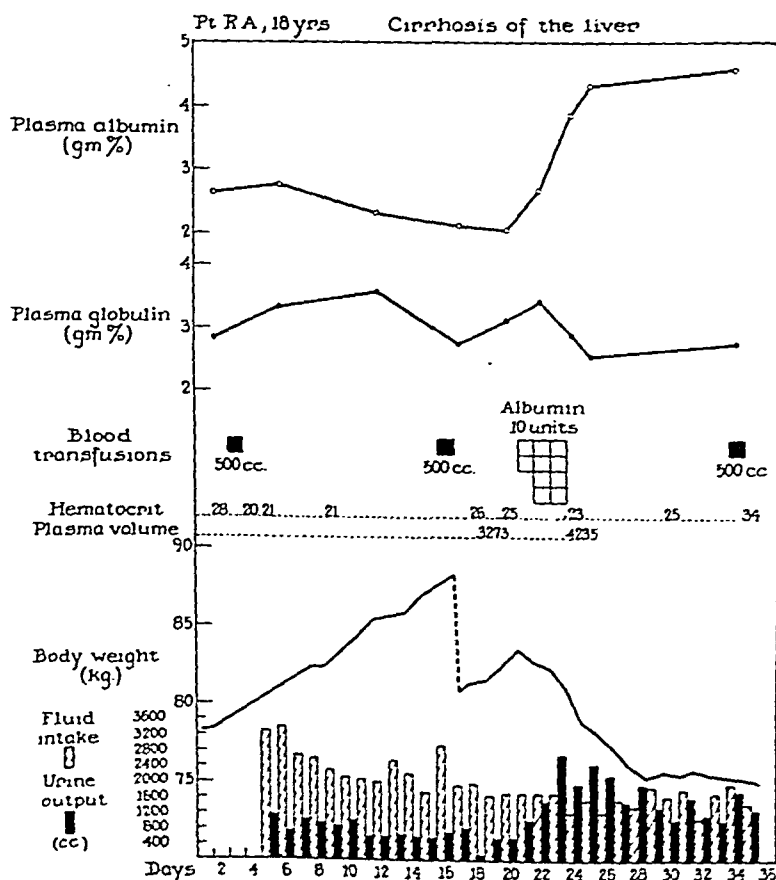
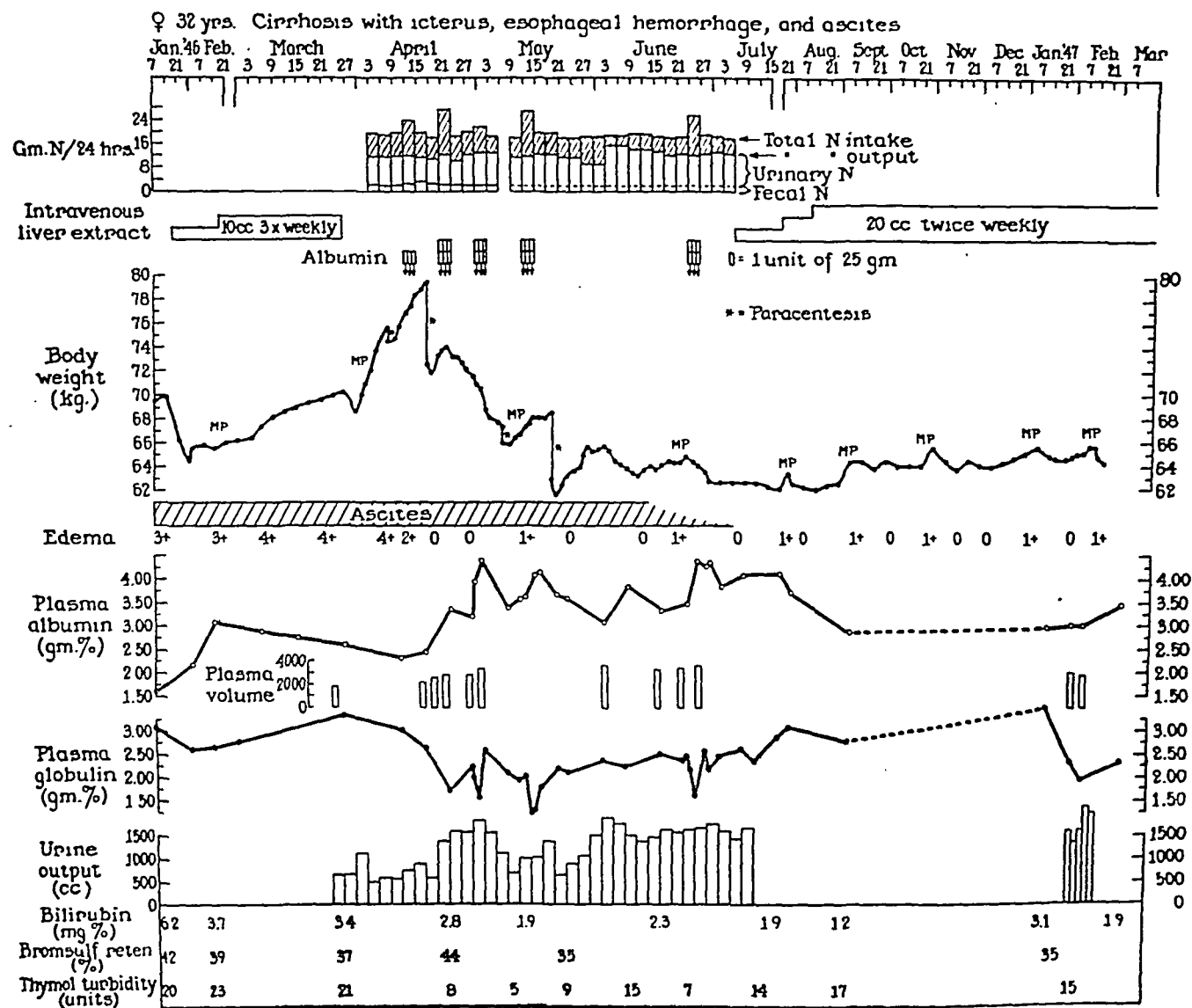


FIG. 1. PATIENT NO. 5 (GROUP I) WITH EARLY ASCITES FOLLOWING AN ESOPHAGEAL HEMORRHAGE

Rapid and complete diuresis following the administration of a small amount of albumin.



Immediate diuresis with loss of edema and delayed diuresis with loss of ascites following albumin therapy.

proved markedly on small doses of albumin. A rapid diuresis was obtained in each case. Esophageal hemorrhages have complicated the course of recovery of two of these patients, one of whom died following a spleno-renal anastomosis.

In each patient in this group albumin administration initiated a more rapid response than could have been expected with dietary and intravenous liver extract therapy. Four of the five patients were able to preserve their artificially elevated serum albumin levels by better synthesis of their own serum albumin.

Group II. This group includes four patients with a nutritional type of cirrhosis all of whom were severely ill with ascites that had been present

constantly for more than five months. Each patient had been given vigorous dietary and vitamin therapy for at least three months prior to the administration of albumin. Three had been started on intravenous liver extract but, because their condition was deteriorating alarmingly, it was believed that a more rapidly acting form of therapy was necessary. This group, therefore, represents patients who would probably have succumbed to their disease despite the use of dietary and liver extract therapy.

Figure 3 illustrates the course of patient No. 6. The condition of this man grew progressively worse in the hospital while he was being given regular injections of liver extract. As may be

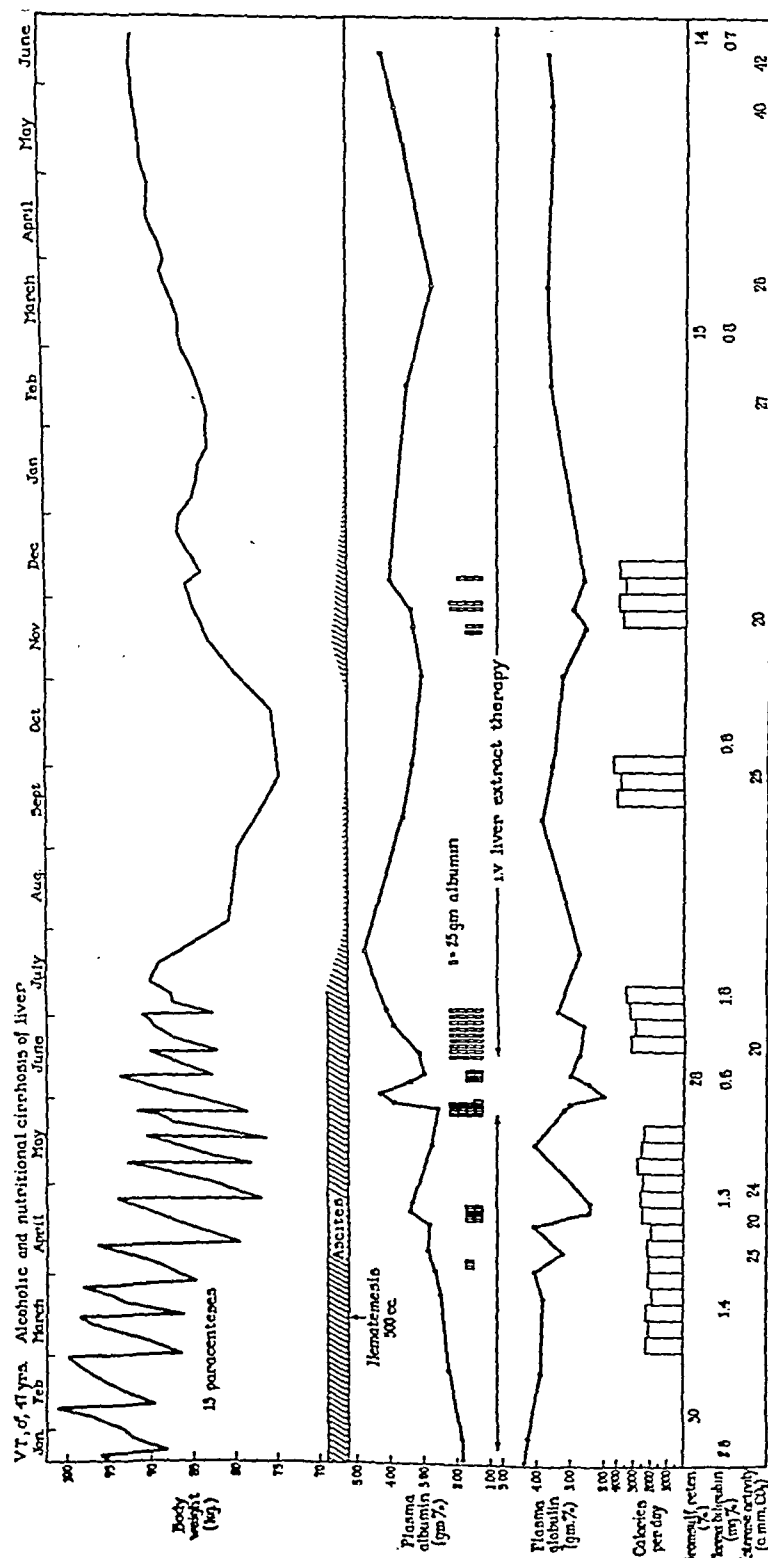


Fig. 3. PATIENT No. 6 (GROUP II) WITH ASCITES OF EIGHT MONTHS' DURATION REQUIRING 30 PARACENTESIS. Early rise of caloric intake following albumin. Markedly delayed diuresis following prolonged therapy with 80 units of albumin. Recurrence of ascites responding to 15 additional units. Return of ability to synthesize albumin one year after initial therapy.

seen from the weight curve, a continuous decline in his basic weight followed each paracentesis. The administration of small amounts of albumin produced little effect. It was not until 4 units were given each day that the serum albumin level was raised to normal and a slight increase in urine volume occurred. The patient also noticed a feeling of well-being and the appearance of appetite that was reflected in an increase in caloric intake. Very little effect was noted, however, on the rate of accumulation of ascitic fluid until 4 units of albumin were administered every other day for more than two weeks. During this time a gradual slowing in the rate of fluid accumulation occurred although no acute diuresis was ever obtained. Therapy was finally stopped after 80 units of albumin had been administered despite the fact that considerable ascites persisted. Approximately two weeks after cessation of therapy a diuresis with loss of all ascites occurred. At the same time the serum albumin rose to the highest level that had been reached. Although his caloric intake was almost double the pre-treatment level, the patient failed to put on body weight. He remained free of fluid for three months but then slowly and despite continued intravenous liver extract therapy reaccumulated ascites until a paracentesis appeared necessary. Instead, 15 units of albumin were administered and a slight diuresis was obtained. Gradually over the next two months all ascitic fluid disappeared spontaneously, and the patient began to put on body weight. The improvement following the second course of albumin was more striking than after the first. The serum albumin, which had gradually fallen following initial therapy, was raised by the second course, but slowly fell once again. He gained approximately 30 lbs. of weight and returned to normal activity. Suddenly, six months after the last albumin treatment, the serum albumin rose spontaneously to normal indicating that the patient had finally regained the ability to synthesize normal amounts of serum albumin. His bromsulfalein retention fell from 30 per cent to 12 per cent over the period of 18 months that he was treated.

The three other patients in the group were treated with large amounts of albumin after failing to improve on other forms of therapy. All but one patient showed a gradual disappearance of ascites and marked clinical improvement. Ascites disap-

peared very slowly, usually several weeks after the patients had begun to feel improved and were eating better. In patient No. 8 moderate ascites recurred three months after therapy, although the patient continued to lead a normal life. Further albumin was not administered. This patient died suddenly because of an intestinal obstruction unrelated to the liver disease. Patient No. 9, who did not respond to albumin therapy, was unusual in that she had been accumulating approximately 20 liters of ascitic fluid every 14 days for nine months prior to albumin administration. The antidiuretic titer was found to be unusually high, approximately twice the normal value (Table III). Eighty units of albumin were administered intravenously over a period of three months without noticeable effect. Although the serum albumin level was kept above 3.5 gms. per cent during this period, paracenteses continued to be necessary every 10 to 16 days.

Group III. Four patients with extremely severe cirrhosis and two patients with fatal subacute infectious hepatitis, all showing albumin levels below 2 gms. per cent, comprise this group. All demonstrated large amounts of edema associated with ascites. Laparotomy biopsy was performed on one patient, and autopsies were obtained on the five patients who died. None of the patients showed a typical Laennec's cirrhosis. The diagnosis was post-necrotic cirrhosis in three patients, biliary cirrhosis in one, and subacute yellow atrophy in two.

All patients demonstrated a diuretic response with disappearance of edema and ascites to an average of 16 units of albumin. Figure 4 illustrates the course of a typical case in this group. The patient (No. 10) was a 32 year old sailor who had had a typical attack of infectious hepatitis 18 months prior to therapy. Following a severe relapse of infectious hepatitis, symptoms and signs of liver damage persisted and he was followed for one year at the Hospital of The Rockefeller Institute. Following discharge from the Navy he attempted full time work, but his symptoms became more severe and he was readmitted to the Rockefeller Hospital with generalized anasarca and ascites. His condition was critical and during a month of dietary and vitamin therapy he required three paracenteses. Following the administration of 15 units of albumin a diuresis with loss

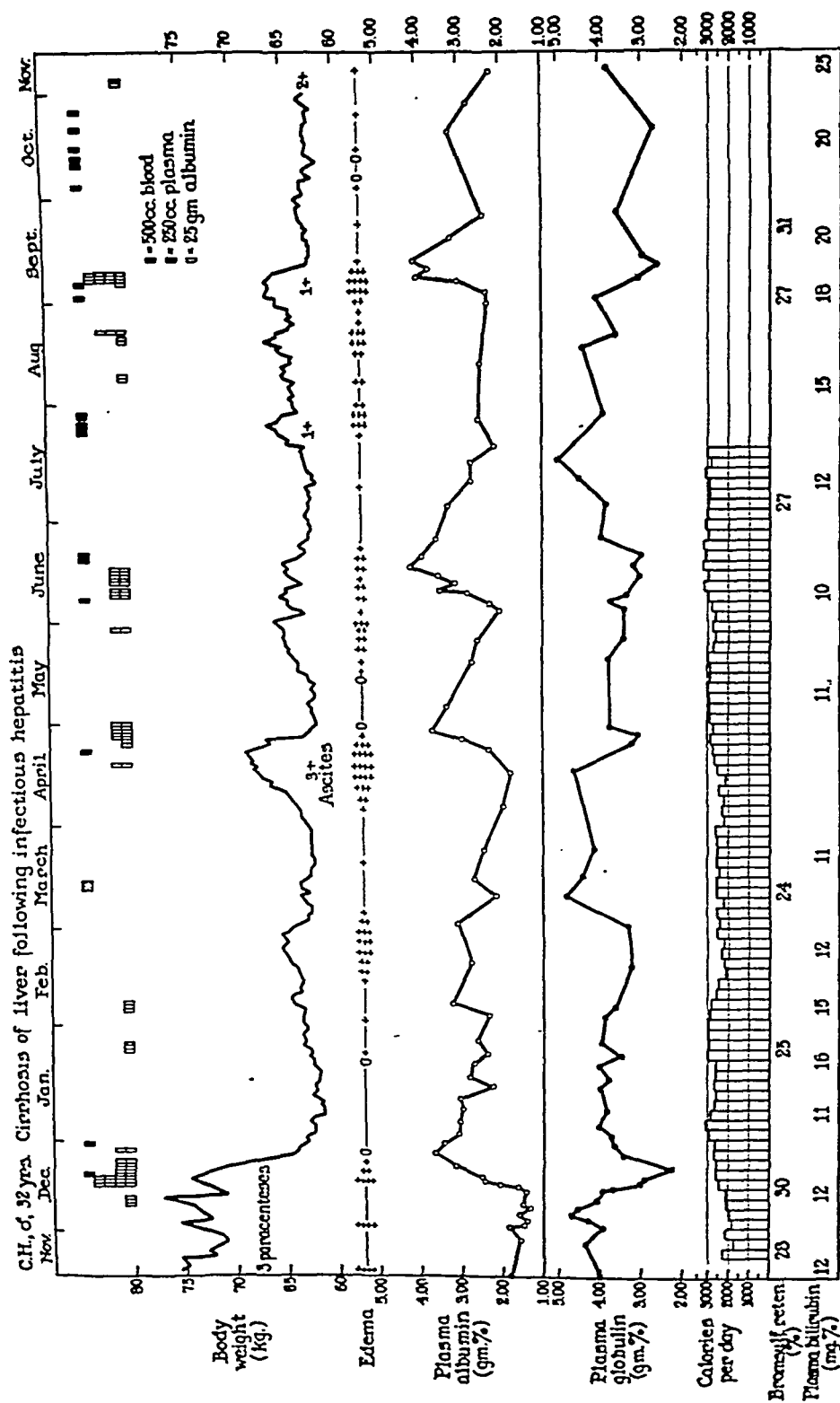


FIG. 4. PATIENT No. 10 (GROUP III) WITH GENERALIZED ANASARCA 18 MONTHS AFTER INFECTIOUS HEPATITIS

Rapid and complete loss of edema and ascites following 27 units of albumin. Control of recurrent ascites with additional albumin. Increased caloric intake with each course of albumin. Death due to uncontrollable bleeding tendency despite control of fluid balance.

of all ascites and edema occurred, and during this diuresis 12 additional units were administered. The serum albumin level was raised to normal and the general condition of the patient improved dramatically. His caloric intake increased from 2,000 to 3,000 calories per day and within two weeks he was able to return to full activity. It was evident that he suffered from a severe albumin deficit which when overcome resulted in marked improvement. Over a period of four months following the first course of therapy the serum albumin level gradually declined and the patient once again developed ascites and edema. The administration of 12 units of albumin produced another immediate diuresis. The patient lived for approximately one year after the onset of albumin therapy, during which time recurring edema and ascites were readily controlled by albumin administration on four occasions. Albumin, however, had no measurable effect in improving his liver function, and the serum bilirubin rose in the terminal four months from 12 mg. per cent to 25 mg. per cent. Synthesis of prothrombin and fibrinogen by the liver were markedly impaired, similar in degree to the albumin deficiency. However, these proteins were not available for replacement therapy, and the patient finally succumbed because of severe, uncontrollable bleeding from his gums and nose despite numerous transfusions with fresh blood. It was believed, however, that the patient's life had been lengthened considerably by albumin therapy.

Two other patients in the group (Nos. 11 and 12) appeared to have had their life span increased by albumin administration. Patient No. 11 received five separate courses of small amounts of albumin with loss of edema and ascites on each occasion. He eventually died during curare treatment for a neurological disorder resembling Wilson's disease. Patient No. 13, who also presented a terminal picture of generalized anasarca with a very low serum albumin, demonstrated a cirrhosis of undetermined etiology associated with severe arthritis. All edema and ascites disappeared rapidly following the use of intravenous albumin. This patient differed from the others in that she continued to preserve the normal serum albumin level brought about by albumin administration and has remained well leading a normal life for 25 months following therapy. Laparotomy biopsy

two years after therapy showed an advanced cirrhosis characterized by dense fibrous tissue strands and large masses of regenerating liver cells. The gratifying response to albumin in this patient emphasizes the difficulty in predicting the therapeutic effect of albumin.

The two patients with subacute yellow atrophy (Nos. 14 and 15) showed a diuresis with loss of edema and ascites while in a semi-comatose state. Their downhill course was unaffected by the albumin therapy and both died. They are additional examples of cases that react readily with a diuretic response to relatively small amounts of albumin.

The factor of portal obstruction was not apparent clinically in this group of patients with severe liver disease. Water retention corresponded closely to the albumin deficit and could be controlled readily by albumin therapy. Although three of the four patients with cirrhosis eventually died, they were markedly improved temporarily by this form of therapy. This effect, together with the dramatic and prolonged improvement in patient No. 13, demonstrates the value of albumin in this type of liver disease.

Group IV. Two patients with post-hepatitis cirrhosis without edema and ascites were given albumin therapy in order to increase dietary intake prior to the expected onset of hepatic decompensation. Patient No. 16 showed a definite increase in caloric intake and was able to maintain his artificially raised albumin level. He gained weight and was able to return to normal activity. Patient No. 17 showed no response. The dietary intake remained poor, marked fatigue persisted, and weight loss continued. However, for seven months he maintained a serum albumin level higher than that prior to therapy.

SPECIFIC EFFECTS OF ALBUMIN THERAPY

The influence of injected albumin on the ability of the liver to resume normal synthesis of albumin is perhaps the most important consideration in evaluating its therapeutic effectiveness. The necessity for periodic injections of albumin in the form of maintenance therapy rests with this question. Table II shows that four of 12 patients were able to form sufficient albumin to maintain an approximately normal level three months after the first course of therapy ended. One other patient (No. 6) regained this capacity following a second

TABLE II

Iterations in plasma albumin level following albumin therapy in 15 patients with cirrhosis of the liver

Albumin level	No. of patients		
	Before therapy	Immediately after therapy	3 mos. after therapy*
3.5 gms. per cent or higher	0	15	4
3-3.5 gms. per cent	0	0	5
3 gms. per cent or lower	15	0	3

* Three patients are not included: two have not been followed for three months following the discontinuation of therapy and one died two months after therapy.

small course of therapy. Three patients experienced a rapid fall in serum albumin after artificial elevation of this level by a series of injections and demonstrated no improvement in their ability to synthesize albumin. The remainder maintained higher levels after treatment but did not show complete restoration to normal. It should be mentioned that the patients who developed low serum albumin levels and ascites several weeks after bleeding episodes were best able to maintain permanently normal levels following therapy.

Nitrogen balance studies were carried out in four of the patients who received albumin. Figure 5 illustrates the results in a typical case. This patient was in slight negative nitrogen balance prior to therapy but then entered marked positive balance during the period of albumin administration. In the four patients studied there was greater than 90 per cent retention of injected albumin nitrogen; they lost ascites following albumin therapy and did not require further paracenteses. As a result, the continued loss of albumin through the peritoneal cavity was obviated. This was not true of the patients who required further paracenteses; they continued to lose albumin nitrogen. Further observations on the patient illustrated in Figure 5 showed that the patient remained in positive balance for at least one month after therapy was discontinued. Figure 5 also illustrates the marked difference between the effect of albumin on the nitrogen balance and of an equivalent amount of nitrogen in the form of casein hydrolysate.

A number of patients volunteered the information that they developed a feeling of well-being

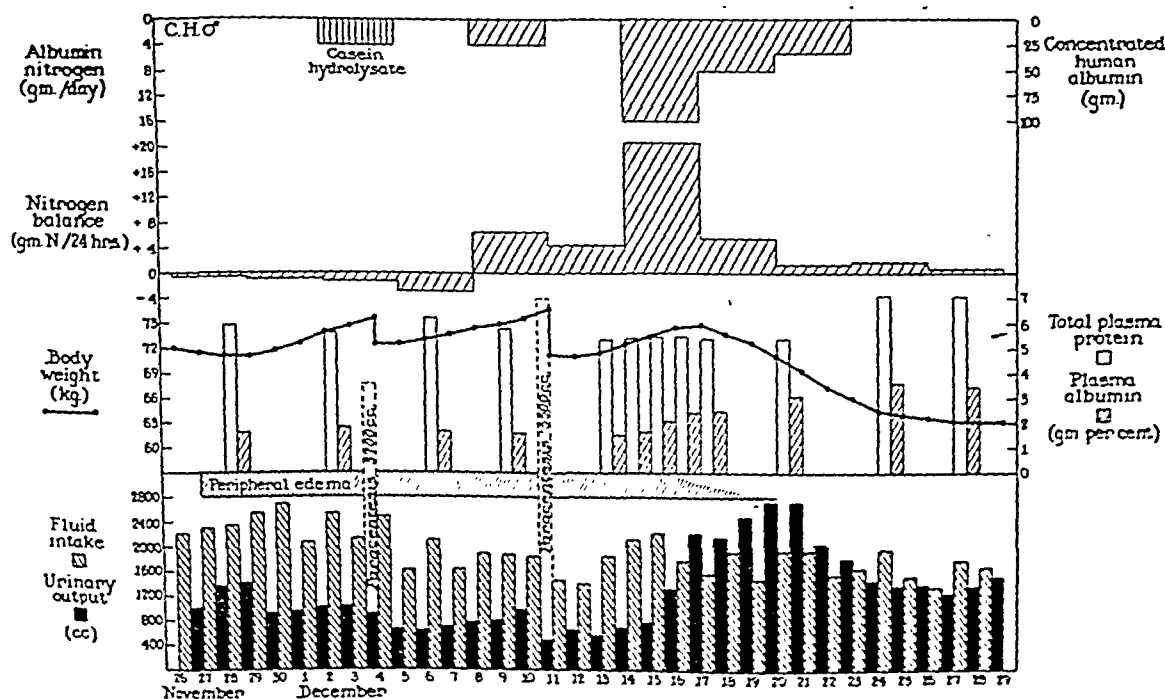


FIG. 5. PATIENT NO. 10 (GROUP III). PERSISTENT NEGATIVE NITROGEN BALANCE WITH CASEIN HYDROLYSATE. Positive nitrogen balance with an equivalent amount of albumin nitrogen. Complete retention of albumin nitrogen.

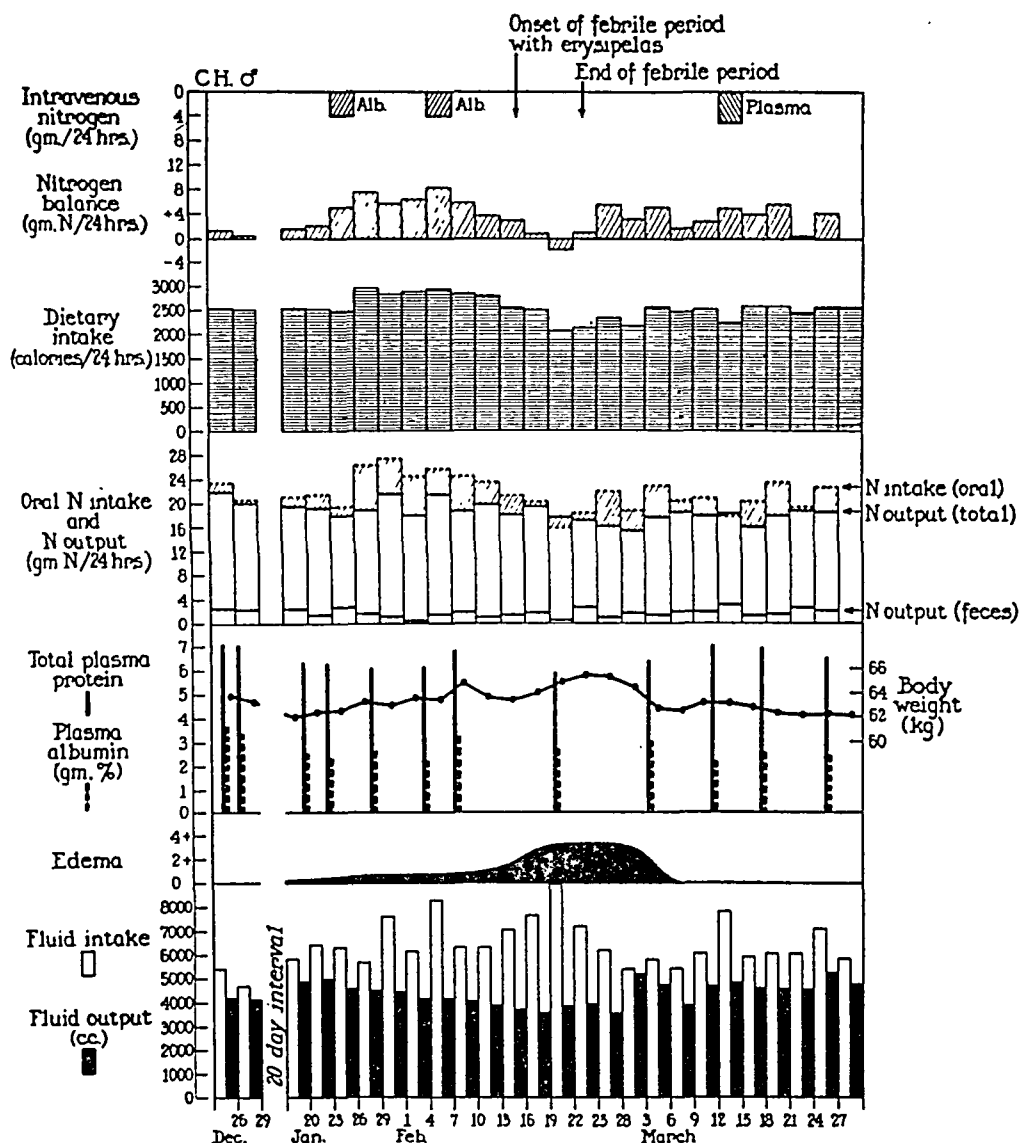


FIG. 6. PATIENT NO. 10 (GROUP III). RISE IN CALORIC INTAKE AND INCREASED RETENTION OF DIETARY NITROGEN FOLLOWING TWO COURSES OF 3 UNITS OF ALBUMIN

following the administration of albumin, despite the fact that no diuresis occurred. This was associated with a general increase in appetite. To test the validity of these observations careful measurements were carried out on the caloric intake before and after albumin therapy in a patient who was free of ascites and showed only minimal edema. Figure 6 illustrates the definite rise in protein and total oral caloric intake following the administration of 3 units of albumin. Nitrogen balance determinations revealed an increased retention of dietary nitrogen in addition to the retention of the nitrogen of the albumin administered. The experiment was interrupted by a short febrile period due to a skin infection. Sub-

sequent administration of plasma did not affect the caloric intake or the nitrogen balance to a similar degree. This specific effect of albumin on the dietary intake was observed during four different periods of albumin therapy in the patient (Figure 4). In another patient (Figure 3) a similar rise in caloric intake during albumin therapy was observed considerably in advance of his diuresis.

Estimation of the degree of retention of brom-sulfalein was carried out before and after albumin treatment in each of the patients. No improvement in this liver function test could be demonstrated within the first month after albumin therapy. However, in four of the patients who were

followed for more than six months following therapy, definite improvement occurred. In patient No. 6 who exhibited no jaundice the bromsulfalein retention fell from 30 per cent to 12 per cent over a period of 16 months. No immediate improvement occurred in serum bilirubin, prothrombin time, total globulin, the thymol turbidity reaction, fibrinogen, and esterase activity following albumin therapy. However, with prolonged treatment for more than six months, changes occurred in these serum components which were in general parallel to the improvement in bromsulfalein retention.

DISCUSSION

The most apparent effect of albumin in patients with liver disease was that due to its osmotic properties. In nine of 15 patients with fluid retention who were treated, a rapid loss of edema and ascites occurred. Five others showed a delayed loss of ascites. In those patients whose diuretic response to albumin was immediate, a beneficial course of events was rapidly initiated; loss of protein into ascitic fluid ceased, dietary protein intake increased, and positive nitrogen balance resulted. Such treatment carried seriously ill patients through the early critical period of their disease until such time as dietary and liver extract therapy had an opportunity to take effect.

The amount of albumin needed to produce a loss of ascites was extremely variable, ranging from 4 to 80 units, and appeared to depend upon at least four factors. The first of these was the relative role of portal obstruction in the causation of fluid accumulation. Since there is no direct method of measuring portal pressure without laparotomy, it was necessary to evaluate this from clinical signs. Esophageal varices and other evidences of collateral circulation, a small hard liver and a large spleen were considered to be associated with portal hypertension. However, the most useful sign was the amount of edema relative to the amount of ascites. Those patients exhibiting marked ascites with little or no edema were considered to have a high degree of portal obstruction, while large amounts of edema in the presence of less impressive quantities of slowly forming ascites indicated that portal obstruction, if present, was of minor importance. Edematous patients with slight evidence of portal obstruction re-

sponded more readily to therapy with albumin than did patients with severe portal hypertension. Patients with subacute yellow atrophy and cirrhosis following infectious hepatitis usually fell into this category. The plasma albumin levels of this group were extremely low, occasionally below 1.5 gms. per cent, suggesting that these patients suffered from a specific albumin deficit. Approximately 12 units, administered over a period of four days, usually produced a dramatic diuresis with loss of edema and ascites. On the other hand, patients with large amounts of ascites in the absence of generalized edema, in whom portal obstruction was considered to be a dominant factor, were generally very resistant to therapy. As much as 80 units of albumin were sometimes necessary before ascites disappeared. It should be emphasized, however, that a diuresis was produced in three out of four such patients with marked evidence of portal obstruction when the plasma albumin was kept at a level of 3.5 gms. per cent or higher for a sufficiently long period of time; the one refractory patient failed to respond to 80 units given over a three-month period despite normal serum albumin levels.

A second factor of importance in determining the amount of albumin required to produce a disappearance of ascites was the rapidity with which the patient accumulated fluid. Following the administration of albumin an equilibrium between blood and ascitic fluid albumin was established, and the A/G ratio in these two body compartments tended to remain equal. As a result, with the rise in albumin concentration of the serum, there was a rise in the concentration of albumin in the ascitic fluid. When the ascitic fluid collection was large and constantly reaccumulating, a sizeable drain on the injected albumin would occur with each paracentesis. Small doses of albumin were ineffective under such circumstances.

The third factor and one which has not been clearly evaluated was the length of time that fluid accumulation had been present prior to albumin therapy. Those patients treated soon after they began to accumulate ascitic fluid responded readily to therapy in each case. The patients most resistant to the diuretic effect of albumin were those who had been accumulating fluid for long periods. The experience gained by treatment of the patients in Group I suggests that early as-

TABLE III

Comparison between the titer of antidiuretic substance in the urine and the amount of albumin required and the type of response produced by albumin therapy in six patients with cirrhosis of the liver

Case no.	Antidiuretic titer	Units of albumin required	Type of diuresis
	<i>minutes</i>		
5	140*	10	Immediate
2	140*	8	Immediate
11	120*	4	Immediate
7	300	24	Very gradual
9	240	80	None
6	160	80	Very gradual

* Normal.

cites is due primarily to low serum albumin levels and that albumin therapy is a specific remedy at this time. In the patients of Group II with long-standing ascites, changes secondary to fluid accumulation have been added to the initial factor of hypoalbuminemia, rendering albumin treatment less effective. Investigations are now in progress regarding the role of alterations of peritoneal absorption in these patients.

The fourth factor influencing the amount of albumin necessary to produce a diuretic effect was the concentration of antidiuretic principle present in the urine of patients being treated. Preliminary observations indicated that those patients excreting large quantities of this substance required prolonged administration of albumin (Table III).

The ultimate effects of albumin therapy are certainly not all directly attributable to albumin alone. In certain patients this material simply initiated a curative process that was then sustained by increased caloric intake and liver extract therapy. A rise in caloric intake and increased retention of dietary nitrogen were demonstrated following albumin therapy in certain patients where the response could not be attributed to relief from the disabling effects of fluid. This effect of albumin was perhaps of even greater significance than its oncotic effects in initiating the process of recovery. In the three patients of Group II who eventually lost their ascites after prolonged albumin therapy, the effects of albumin on food intake and utilization may have been primarily responsible for the eventual disappearance of ascites. The fact that these patients demonstrated

a delayed diuretic effect after albumin therapy raises the question whether supplemental nutritional and liver extract therapy which the patients were receiving continually might have been primarily responsible for improvement. However, against this possibility the following observations may be cited. First, during control periods on supplemental treatment alone these patients had failed to improve. Secondly, increased appetite and a general feeling of well-being were closely associated in time with albumin therapy. Finally, the most conclusive evidence for the primary role of albumin was obtained in those patients (Nos. 6 and 13) who developed a recurrence of ascites several months after the first period of therapy and in whom another small course of albumin caused improvement and loss of ascites for the second time.

SUMMARY

1. The results of serum albumin therapy in 17 patients with severe liver disease are presented.
2. Fourteen out of 15 patients with ascites lost their fluid following therapy.
3. The amount of albumin necessary to produce such an effect was variable ranging from 4 to 80 units.
4. Patients with marked evidence of portal obstruction, a high antidiuretic titer in the urine or long-standing ascites proved very resistant to therapy.
5. Patients with particularly severe liver disease and very low plasma albumin levels following infectious hepatitis responded most readily to therapy. Cases of alcoholic and nutritional cirrhosis with a short period of ascites also responded to relatively small doses of albumin.
6. Permanently beneficial results were obtained in six of seven patients with the nutritional type of cirrhosis. Two of these patients required a second short course of therapy. Patients with post-necrotic cirrhosis after infectious hepatitis and biliary cirrhosis showed only a temporary response and four of these patients eventually died.
7. Evidence was presented for certain specific effects of serum albumin on dietary intake and nitrogen balance which may be of greater importance in the results obtained than its osmotic properties.

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THE COMPLEMENT CONTENT OF HUMAN SERA WITH ESPECIAL REFERENCE TO MALARIA¹

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Our interest in the complement content of sera from healthy and diseased individuals was initiated by chance observations on the cephalin-cholesterol flocculation test (1). In our first performance of the test we inadvertently included several sera which had been inactivated and stored in the refrigerator over-night. All of these gave positive readings at 24 hours. Repetition of these tests, using sera from the same individuals, but fresh samples which were less than four hours old, yielded some negative and some positive reactions.

It appeared that heating sera at 56° C. for 30 minutes might have played a part in the non-specific reactions with the cephalin-cholesterol antigen. Sera from 20 healthy individuals were therefore tested before and after inactivation. None of the 20 sera gave positive readings before inactivation, whereas five gave readings after heating which would be considered significant.

These results demonstrated that certain normal sera may be altered by inactivation to the extent that they give false positive cephalin-cholesterol reactions. The mechanism of inactivation is not altogether understood. One important result has to do with the destruction of the hemolytic property of the serum. Denaturation of proteins may occur and there may be loss of stabilizing property. However, determination of the complement content of these 20 sera, according to methods described later in this report, revealed no differences which could be related to the cephalin-cholesterol flocculation test. Later it was found that the addition of varying amounts of complement or albumin to positively reacting sera, or to normal sera which gave positive reactions after inactivation, did not inhibit the positive reaction.

In review of papers regarding the basic principle of the cephalin-cholesterol reaction, it was found that Kabat *et al.* (1943) (2) had observed the same non-specific reactions given by certain sera after inactivation and ice-box storage. They removed the complement from two normal sera by means of the precipitate formed by addition of purified rabbit Type III antipneumococcus nitrogen and Type III polysaccharide without development of a positive cephalin reaction.

These preliminary observations led us to study the complement content of sera from normal and diseased individuals. The recent papers of Wassermann and Alberts (1940) (3), Rutstein and Walker (1942) (4), Traub (1943) (5), Pohl and Rutstein (1944) (6), and of Ecker *et al.* (1946) (7, 8) demonstrate the renewed interest in the subject of complement in various clinical states. Ecker and his group have reviewed the considerable series of papers which have to do with complement in infectious diseases of man. They suggest that the lack of uniform technic explains in part the marked "contraindications and inconsistencies" of data. In their study of complement in 278 cases of various infectious diseases they determined the titers of the individual complement components as well as the inclusive titers. All other reports, including this one, record the overall titers.

We were particularly interested in the complement content of malarious sera and in the possible correlation with parasite count and other laboratory and clinical data. No complete data on complement in human malaria are available, although the papers of Cathoire (1910) (9), Vincent (1910) (10), Radosavjevic (1923) (11), and Wendlberger and Volavsek (1934) (12) state that complement is reduced in malaria and that such a decrease is most marked after several paroxysms. Roy and Mukerjee (1942) (13) re-

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port that complement is diminished in monkey malaria of *P. knowlesi* origin.

In this laboratory the amount of complement in milliliters required for 50 per cent hemolysis was determined for the following groups: (1) 30 normal healthy individuals, (2) 25 patients with liver disease, (3) 32 persons with various types of non-infectious disease, and (4) 24 neurosyphilitics before and after induced malaria. Single samples of sera were in most instances tested for the first three groups while multiple determinations were made on all but three of the patients inoculated with malaria. In all, a total of 264 determinations of complement titer were performed on 111 individuals.

METHODS

For the determination of the 50 per cent unit of complement the method of Kent, Bukantz and Rein (14) was followed with slight modifications.

Collection of sera: With the exception of a small number of malarious sera, collected at different times during the paroxysms, all of the bloods represented early morning samples (8 a.m.) from fasting individuals. The sera were stored at 4° C. until the titrations were carried out. All titrations were performed within a period of four to five hours after collection of the blood samples.

Complement titration: Sera were routinely titrated in two dilutions. In most instances dilutions of 1:30 and 1:40 yielded satisfactory results. With sera showing diminished complement it was necessary to employ dilutions of 1:20 and 1:10, or even zero.

A 2 per cent suspension of sheep red cells was used. The blood was obtained from the same group of sheep, each of which had been tested as to red blood cell count and suitability for use in hemolytic systems. The blood was collected each week in Alsever's solution.² On the day of use the citrated blood was filtered through gauze into a 15-ml. centrifuge tube and centrifuged. The packed cells were washed three times with physiological saline, all centrifugations being carried out at 2000 r.p.m. for ten minutes. A 2 per cent cell suspension was prepared, and after thorough mixing 1.2 ml. were pipetted to each of two Klett tubes and 4.8 ml. of cold distilled water added. After centrifugation at 2000 r.p.m. for five minutes readings were made in the Klett-Summerson colorimeter, using a green filter. The red cell suspension usually read at, or close to, 350.³ Adjustments to this figure were made by removal or addition of saline to the stock suspension, according to the formula

$$Vol_2 = \frac{Vol_1 \times \text{Klett reading}}{350}$$

² As described (14).

³ This reading has been found to correspond with a cell concentration of 500,000 per cu. mm.

The adjusted suspension was checked in the manner described above.

The same lot of stock hemolysin was used throughout these studies. Titrations of diluted hemolysin were made at intervals to insure use of the optimal unit (15). One such unit of hemolysin in an equal volume of physiological saline was used for sensitization of the sheep red cell suspension.

Klett tubes were used for the complement titrations and Table I indicates the amounts of the reagents employed.

TABLE I
Amounts of reagents (in ml.) employed in titration of complement

Tubes	1	2	3	4	5	6	100% Con- trol	0 Con- trol
Diluted serum	0.6	0.75	0.9	1.05	1.2	1.35	0	0
Saline	3.0	2.85	2.7	2.55	2.4	2.25	0	3.6
Sensitized cells	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Cold distilled water	0	0	0	0	0	0	3.6	0

After an incubation period of 30 minutes in a 37° C. water bath the tubes were centrifuged at 2000 r.p.m. for 10 minutes and examined in the colorimeter. The percentage of hemolysis was determined for each tube by comparison with the 100 per cent hemolytic and negative controls. A conversion table (Kent) was employed for adjustment and the average of readings in the 20–80 per cent hemolytic range recorded. This average reading divided by the figure representing the serum dilution gave the volume in milliliters of complement required for 50 per cent hemolysis.

RESULTS

Normal healthy individuals: The 50 per cent complement titer of this group of 30 ranged from 0.0032 ml. to 0.006 ml. Six individuals yielded titers of 0.003–0.0039 ml.; 15, 0.004–0.0049 ml.; eight, 0.005–0.0059 ml. while one titer exceeded 0.006 ml. The average titer was 0.0045 ml.

These data correspond very closely to those of Rutstein and Walker (4) for 54 healthy individuals. They followed the technic of Wadsworth, Maltaner and Maltaner (16) for the determination of the volume of blood serum in milliliters required to produce 50 per cent hemolysis and report a range of 0.0029–0.006 ml. Forty-two (80 per cent) of their group gave values of 0.004–0.0059 ml. In our series, 23 (77 per cent) of the 30 patients showed values of 0.004–0.0059 ml. In a subsequent paper Pohl and Rutstein (6) report observations on 71 normal individuals and state

that the median amount of blood serum required for 50 per cent hemolysis was 0.0049 ml. This median was not related to age or sex. As stated above our median was 0.0045 ml. and this was not related to sex. There were no significant age differences.

Day-to-day variation in the complement content of healthy normal individuals appears to be slight. For example, we drew blood from B. E. for eight successive days. All samples were collected at approximately 8 a.m., prior to breakfast. The 50 per cent hemolytic units in milliliters were consecutively: 0.0042, 0.0041, 0.0038, 0.0043, 0.0041, 0.0046, 0.0039, and 0.0041. The slight differences in these values are regarded as insignificant.

Patients with liver disease: The 25 patients in this group included five with a diagnosis of acute infectious hepatitis; seven, portal cirrhosis; five, Laennec's cirrhosis; two, carcinoma of the liver; two, chronic alcoholism and four, miscellaneous classifications. Only one sample of serum was examined for 21 patients while four patients had two examinations each. The 50 per cent unit for one of these individuals read below 0.002 ml. while six read 0.002-0.0029; 12, 0.003-0.0039; four, 0.004-0.0049; two, 0.005-0.0059; and four over 0.006 ml.

These data suggest that even severe disease of the liver does not produce diminution of complement detectable by such random sampling. It is possible, of course, that had these patients been followed for a period of several weeks certain ones would have shown marked variation in their complement titers. It is our opinion that a diminution of complement is a part of the picture in all severe disease which threatens to overwhelm the host.

Patients with non-infectious diseases of various types: This group included 32 patients on whom 34 examinations were made. When these titers are compared with those of the normal group, or with those of patients with liver disease, it is apparent that there are no significant differences. Eight individuals gave titers of 0.002-0.0029 ml.; 20, 0.003-0.0039 ml., while there were two representatives in each of the groups, 0.004-0.0049, 0.005-0.0059 and over 0.006 ml.

The average 50 per cent hemolytic titer for this group is 0.0036 ml., a figure which almost dupli-

cates that of 0.0037 ml. for the patients with liver disease. Both of these medians are somewhat higher⁴ than that for the normal group (0.0045 ml.) but it is not believed that the difference, as based on one test, justifies the conclusion that complement is not decreased in the group of patients with non-infectious disease.

Patients with malaria: A total of 178 complement titrations were performed on 24 malarious patients. One observation was recorded for each of three patients and two to seven tests for each of 15 patients. A total of 112 titrations were carried out on six patients. One of these individuals had 11 tests; one, 12 tests; and one, 17 tests. Twenty-three successive titrations were conducted on each of two patients and 26 on another.

The distribution of complement titers in relation to the presence or absence of parasitemia in the individual samples of blood from the 24 malarious patients is presented in Table II.

TABLE II

The distribution of 178 complement titers for 24 malarious patients in relation to the presence or absence of demonstrable parasitemia

Ml. of serum	No parasitemia	Parasitemia present	Total
0.002-0.0029	1	4	5
0.003-0.0039	19	20	39
0.004-0.0049	24	20	44
0.005-0.0059	8	23	31
0.006-0.0069	1	9	10
0.007-0.0079	1	5	6
0.008-0.0089	1	3	4
over 0.009*	16	23	39
Total	71	107	178

* These titers were obtained with sera from patients P. W. and M. A. only.

Statistical analysis of these 178 titrations, considered as isolated observations, revealed no significant difference between the amount of complement required for 50 per cent hemolysis in bloods exhibiting parasitemia and in those free of parasites. There was likewise no correlation between the complement titer and the degree of parasitemia since high and low parasite counts are represented in all groups.

However, a study of the composite records of

⁴ The activity of complement is inversely related to volume.

TABLE III

Pertinent clinical and laboratory findings for six patients with induced malaria

Patient	Type of malaria	Period of observation (days)	Number of tests	Days between tests	Complement titer before inoculation	Lowest titer during malaria	Days after onset of parasitemia	Number of paroxysms	Hours of fever*	Cephalochlocculation	Clinical course
1	<i>P. falciparum</i>	31	23	1-2	0.0045 ml.	0.01 ml.	8	5	34	0	uneventful
2	<i>P. vivax</i>	18	11	1-2	0.0056 ml.	0.043 ml.	3	1	8	+	severe
3	<i>P. vivax</i>	33	23	1-2	0.0048 ml.	0.28 ml.	15	11	51	+	severe
4	<i>P. vivax</i>	40	26	1-2	0.0039 ml.	0.25 ml.	15	4	13	+	severe
5	<i>P. falciparum</i>	25	17	1-2	0.0046 ml.	0.004 ml.	11	5	23	0	uneventful
6	<i>P. vivax</i>	67	12	3-7	0.0034 ml.	0.005 ml.	16	6	54	+	uneventful

* 103° F. by rectum.

patients proves that complement is usually depressed during induced malaria. This diminution may be so slight that the lowest titer of some patients is still within the range exhibited by certain normal individuals and in such cases it may be demonstrated only by a series of examinations made at short intervals. Other patients may show marked decrease of complement. In general, complement decrease is a part of the picture of severe disease.

Since it is believed that the data on the six patients followed over relatively long periods are most informative, these will be considered in detail. Table III gives the pertinent clinical and laboratory data, and Figures 1-6 show the trend of complement and parasitemia and indicate the significant changes which occurred after onset of parasitemia.

The individual records of eight patients on whom at least five titrations were performed are

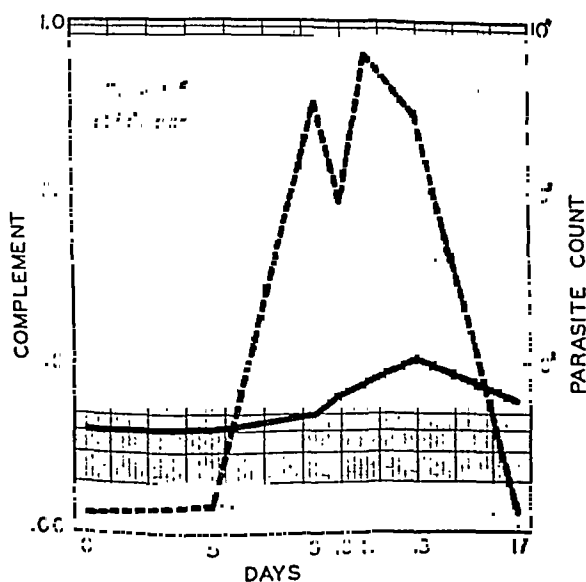


FIG. 1

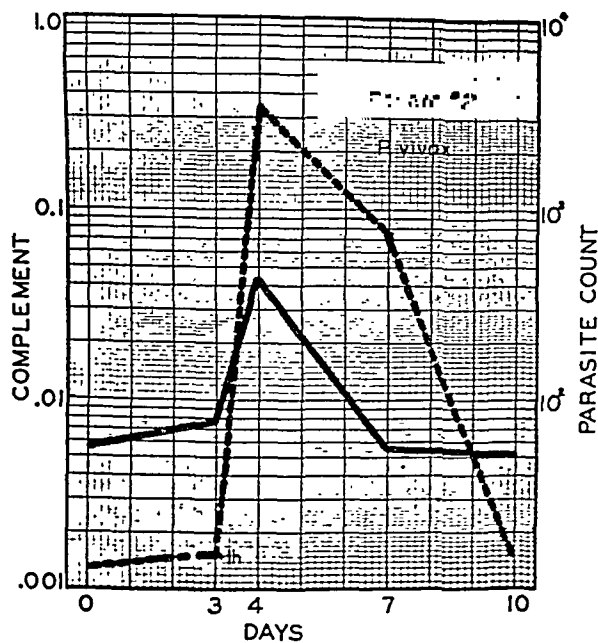


FIG. 2

FIGS. 1-6. SHOWING RELATIONSHIP OF PARASITE COUNT AND COMPLEMENT CONTENT OF SERA OF SIX PATIENTS WITH INDUCED MALARIA. THE AMOUNT OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS IS INVERSELY PROPORTIONAL TO TITER

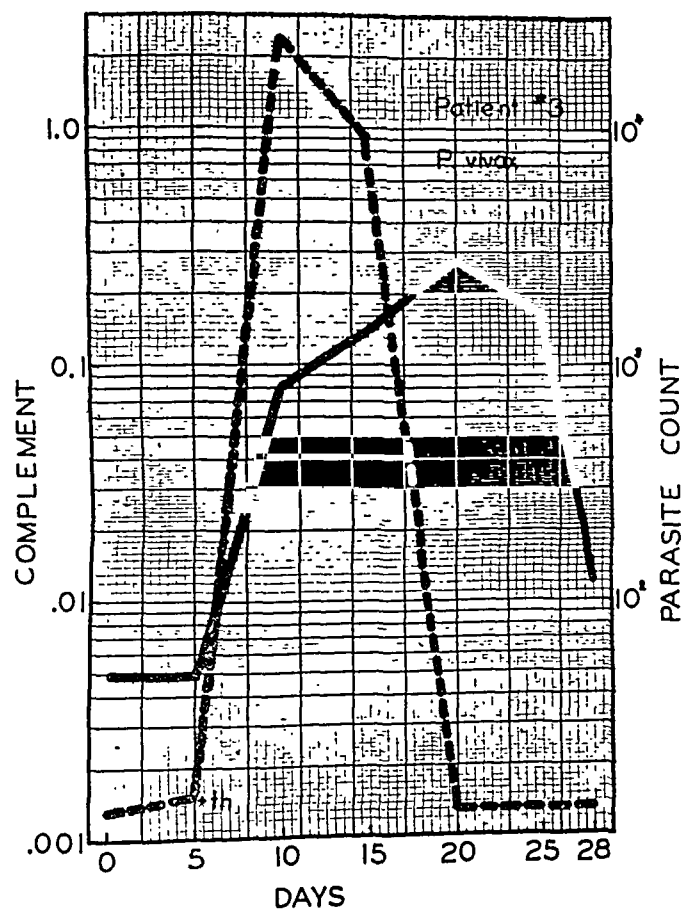


FIG. 3

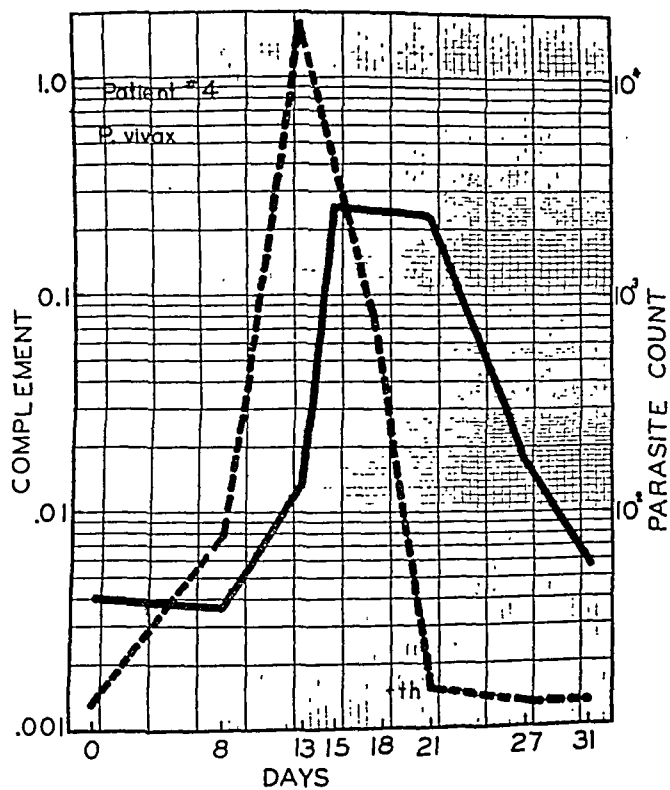


FIG. 4

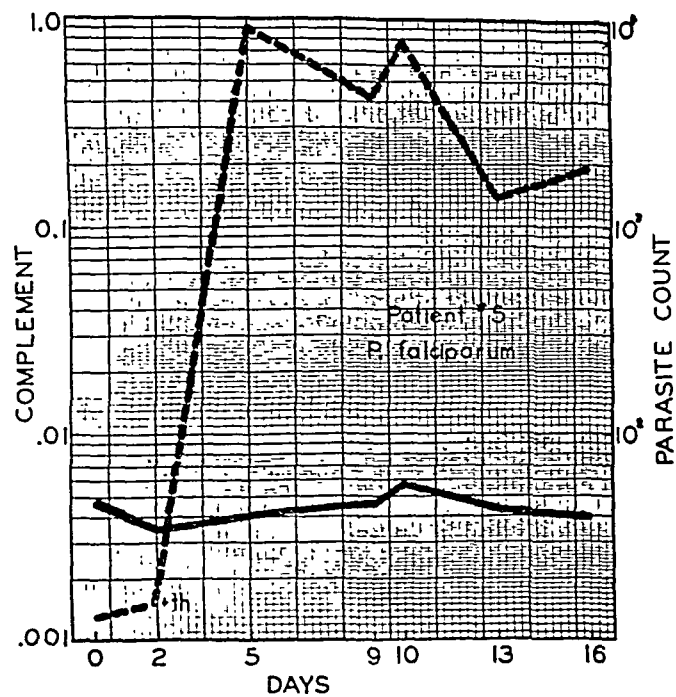


FIG. 5

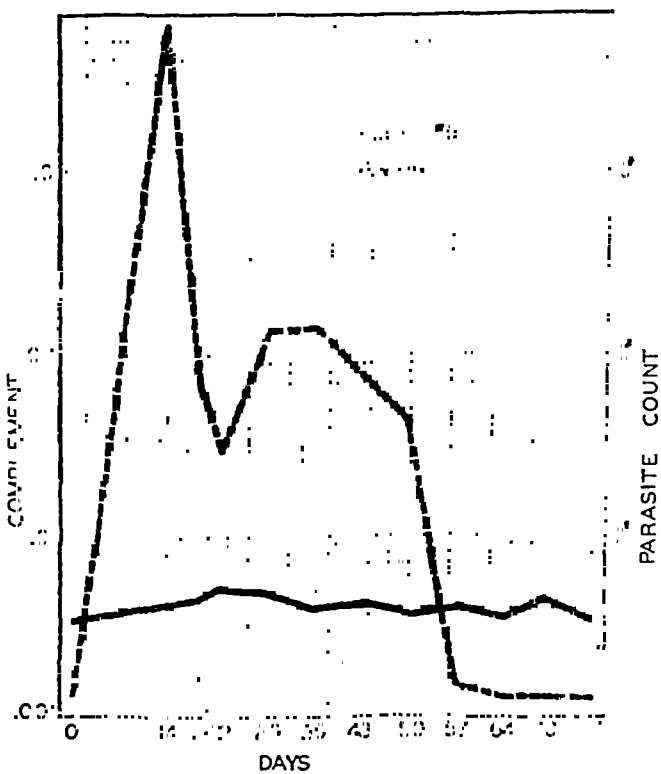


FIG. 6

not included because of lack of space. It is emphasized that all eight followed the general pattern exhibited for malaria. There was a decrease in complement which coincided with, or followed, the peak of parasitemia. In none of these patients was the diminution of complement marked, or the duration of low level prolonged.

In none of the 24 patients was the titer before inoculation with malaria of a level significantly lower than that of the normal group. It is believed that the complement decreases were altogether due to malaria and that late syphilis could not be held responsible for the changes. It is recognized that similar studies in a non-syphilitic group are necessary for a dogmatic statement regarding this question.

DISCUSSION

We are not able to offer an explanation for the depression of complement which usually occurs in induced malaria. There is no doubt that the absolute decrease is correlated with parasitemia (Figures 1-6) since diminution of complement coincided with, or followed, the peak of parasite count. However, the relative decrease in complement cannot always be related to the height of the parasite count. For example, Patient VI exhibited the highest parasitemia of any patient with no marked changes in complement level. On the other hand the high parasite counts of Patients III and IV were accompanied by marked depression of complement. It is interesting that Rutstein and Walker could not relate pneumococcal bacteremia to low complement levels.

The lack of correlation between the numbers of parasites in the blood stream and complement titers would tend to refute any suggestion that complement was decreased as a part of the phagocytic process which is such an important mechanism in malaria.

It is well known that profound disturbances in serum proteins may occur during the course of malaria. There is a marked increase in gamma globulin with a relative decrease in albumin. Davis, Kabat, Harris, and Moore (17) have shown that the gamma globulin fraction of human serum separated by electrophoresis is often anticomplementary and they point out that sera from patients with certain infectious diseases characterized by marked increase in gamma globulin have

been found anticomplementary. Parenthetically we may state that anticomplementary reactions were not infrequently encountered in sera from our group of malarious patients in complement fixation tests for malaria and syphilis. The possibility of *in-vivo* binding of complement by gamma globulins is interesting to consider. If this were the complete explanation, however, we would expect to demonstrate some correlation between depression of complement and a positive cephalin reaction. Our data do not point to this correlation. While it is true that the patients with severe malaria and coincident complement depression exhibited a positive cephalin test we may cite in contrast the patients with liver disease and a positive cephalin reaction whose blood serum showed high normal levels of complement.

Morrison, Block, and Jeskey (18) analyzed the plasma proteins of monkeys infected with *P. knowlesi* malaria. They found that the gamma globulins were increased at the expense of the albumins and that the A/G ratio was correlated with duration of infection. In the malarious patients studied we could find no such relation between the low complement titer and duration of the disease. We were likewise unable to relate the complement titer to clinical or laboratory findings such as temperature, number of paroxysms, hours of fever, white blood cell count, cephalin flocculation test, or antibody titer.

At this time we can only emphasize that complement depression may be a part of the picture of malarial disease. It is our opinion that no simple explanation of this depression will suffice.

CONCLUSIONS

1. The over-all complement content of normal human sera (as based on the amount in milliliters required for 50 per cent hemolysis) is remarkably constant.

2. The 50 per cent hemolytic unit for normal individuals, as determined by the method employed by us, ranged from 0.0032 to 0.006 ml. with a median of 0.0045 ml.

3. Day-to-day variations in the complement content of normal individuals appear to be slight.

4. The complement level of sera (one sample) from patients with liver disease and from patients with non-infectious diseases of various types did

not appear to be depressed. There was no correlation between complement titer and the results of the cephalin-cholesterol test.

5. Complement is usually diminished during the course of induced malaria. This decrease may be very slight or in severe disease it may be very marked. In general the complement titer reflects the severity of the disease, the balance between host and parasite. Complement titer cannot be correlated with parasite count, white blood cell count, temperature, number of paroxysms, hours of fever, cephalin flocculation test, or antibody titer.

6. In any study of complement as related to a particular disease, it is important that a series of determinations be carried out at short intervals during the course of the disease in order to detect changes which may occur.

ACKNOWLEDGMENTS

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LUNG FUNCTION STUDIES. I. THE RATE OF INCREASE OF ARTERIAL OXYGEN SATURATION DURING THE INHALATION OF 100 PER CENT O₂¹

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It has been noted (1, 2, 3) that the rate at which arterial oxygen saturation (measured by the oximeter) rises during the inhalation of 100% oxygen is considerably slower than expected, if complete saturation of hemoglobin occurs at a pO₂ of 159 mm. Hg at 37° C., as is commonly believed (4, 5). We have been impressed by the finding that otherwise normal men, made anoxic by the inhalation of 10% O₂, attain 100% oxygen saturation of arterial hemoglobin practically as quickly as do normal men previously breathing room air, if both groups be given 100% O₂ to breathe. In both groups the rise in saturation from either 70% or 95% is very rapid until 98–99% saturation is reached but the attainment of the last 1–2% in all subjects is a much slower process.

The studies to be reported here represent an attempt to analyze the possible causes of the slow rise in arterial oxygen saturation that occurs in the extreme upper portion of the dissociation curve. Several explanations may be advanced for this phenomenon: (a) delayed ventilation may occur in some alveoli through which blood is circulating (2); (b) alveolar pO₂ may not be raised promptly enough even in well-ventilated alveoli; (c) the arterial pO₂ may not be in equilibrium with the alveolar pO₂ during periods involving rapid and large changes in alveolar pO₂ (1); (d) venous-arterial shunts, bypassing the pulmonary circulation, may exist; (e) incomplete mixing of arterialized blood may occur; (f) the oximeter may not be analyzing pure arterial blood but blood from which some oxygen has

been extracted by the metabolism of ear tissues; or (g) hemoglobin may require exposure to oxygen tensions considerably in excess of that in room air (159 mm. Hg) to achieve complete saturation.

The simultaneous use of the nitrogen analyzer and oximeter to record continuously the nitrogen content of respired air and oxygen saturation of arterial blood, respectively, has yielded data which permit us to analyze some of these factors more closely.

METHODS

1. Forty-five healthy white male subjects (medical students and physicians) between the ages of 19 and 31 were studied.

2. Arterial oxygen saturation was measured with the automatically compensated oximeter (6). Oximeter saturation changes were observed visually and recorded by 0.25% intervals; this was permitted by a narrow galvanometer beam. The instrumental lag to complete deflection is about six seconds. After the start of O₂ inhalation, saturation readings were made at intervals of ten seconds for two minutes, and at 30-second intervals thereafter until five minutes had elapsed. Readings taken at intervals of five seconds for the first 90 seconds on 17 subjects showed that no significantly different rate of increase was obtained by reading at the shorter time intervals. Ear thickness readings were checked at 130 seconds and five minutes to note any change in vasodilation. In some subjects, oximeter saturation readings were made at 30-second intervals for two minutes after the subject resumed breathing room air.³

³ Calibration studies of the oximeter readings against direct arterial blood analyses have estimated the oximeter error at not over 5%. However, the accuracy of our measurements cannot be assessed by saying that the oximeter is accurate only within 5%. First, it is pointed out by Hemingway and Taylor (7) that, in their method of testing, agreement of better than 5% is not possible, due to certain random errors inherent in the method of testing which would account for discrepancies of 2–5% even if the oximeter gave the correct instantaneous saturation value. Secondly, for assessing the significance of small changes, we feel that these changes are best compared with the variability observed during the control periods before and after the test procedure.

¹ The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Pennsylvania. Under the terms of this contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

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3. Analysis of the respired air was made continuously with a nitrogen meter (8, 9). This instrument continuously samples, analyzes and records the nitrogen content of constant composition samples with an accuracy from day to day of $\pm 2\%$ N_2 relative to Haldane analyses. This is an overall accuracy summing errors in sampling methods, measurement of records and setting the instrument. However, the accuracy for changes in gas composition over short time periods is probably better. The instrumental delay between the time the sample enters the analyzer and the initial change in the record is about 0.04 second. To a stepwise change in composition, the instrument requires approximately 0.07 second to record 95% of the final value.

The sampling needle was placed in the middle of the mouthpiece lumen 1-2 cm. external to the subject's lips. Continuous analysis was made of the respired gases for the first two minutes after inhalation of O_2 , and one to two breaths were analyzed after five minutes on O_2 . After the subject resumed breathing room air following the five-minute period on O_2 , continuous analysis of the respired gas was made for the first two minutes.

4. *Breathing apparatus.* A rubber mouthpiece and nose clip were used. Subjects breathed through a four-way valve of 2 cm. internal diameter. Room air was breathed through one orifice, a dead space of 40 cc. being thus added. With this orifice closed, the subject inspired 99.6% oxygen from a high pressure supply tank through a demand regulator, and exhaled through a Sudd valve to room air. When the subject breathed oxygen, the apparatus dead space was 60 cc. When gases other than O_2 were breathed, these were stored in high pressure tanks and supplied to the subject by a similar demand regulator connected to the mouthpiece via a three-way stopcock.

5. *Procedure.* Subjects were not in basal condition, but sat quietly in a chair for ten to 15 minutes before inhaling oxygen. During this time the oximeter ear unit was placed on the ear. After "ear thickness" readings

became stable, the subject breathed room air through the mouthpiece and the saturation reading was arbitrarily set at 95-96%. If breathing was quiet and regular, this reading was very stable. After the saturation reading was made with the subject breathing room air, the recording camera of the nitrogen meter was started, the room-air orifice in the four-way valve was closed during an expiration, and the start of the following inspiration of oxygen was timed with a stop watch. The hissing noise of the demand regulator served as a convenient signal of the start of inspiration. Thereafter oximeter and nitrogen analyzer readings were made as stated above. Throughout the procedure the subject breathed quietly in a sitting position.

RESULTS

1. *Arterial oxygen saturation increase.* Figure 1 shows graphically the manner in which arterial oxygen saturation increases. After an average delay of 7.1 seconds following the start of the first inhalation of oxygen, saturation begins to increase. This delay represents the interval between the times that O_2 enters the mouth and more fully oxygenated blood reaches the ear. As such it measures the time required for movement of O_2 to the alveoli, for diffusion of O_2 across the alveolar membrane, and the circulation time from lung to ear capillaries. Data obtained in this study indicate that inhalation of O_2 is a more satisfactory and less hazardous method for estimating "lung to ear" (actually "mouth to lung to ear") circulation time than is inhalation of a deep breath of N_2 , a method used by Wexler and Whittenberger (10).

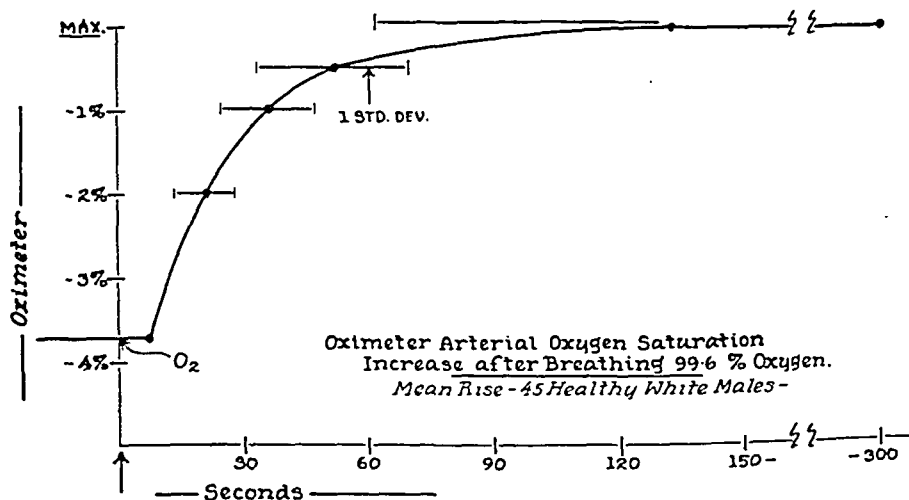


FIG. 1. RATE OF INCREASE OF ARTERIAL OXYGEN SATURATION (OXIMETER) DURING THE CHANGE FROM INHALATION OF AIR TO 99.6% OXYGEN

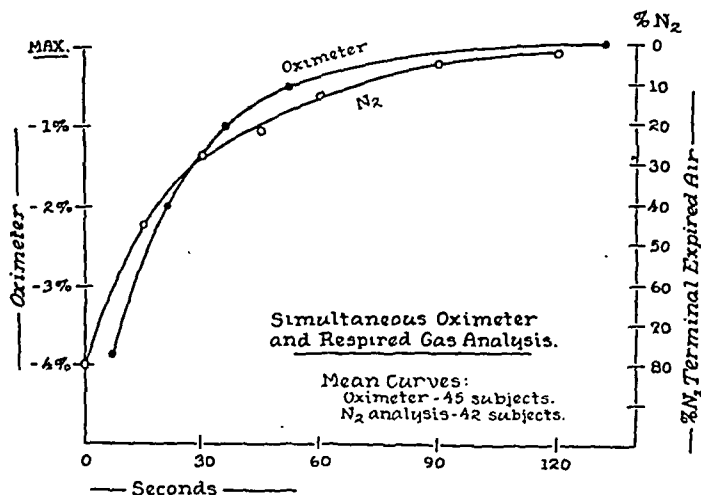


FIG. 2. SIMULTANEOUSLY DETERMINED RATES OF INCREASE OF ARTERIAL OXYGEN SATURATION AND DECREASE OF CONCENTRATION OF NITROGEN IN THE EXPIRED GAS DURING THE INHALATION OF 99.6% OXYGEN

After this delay, the saturation increases rapidly, and then more slowly until a relatively stable plateau has been reached. Minor fluctuations not exceeding 0.5% may occur about this plateau. They are of two types. One type is irregular and probably instrumental. The other is rhythmic and phasic with respiration, seen both on saturation and ear thickness oximeter readings in some persons; it probably indicates slight variations in blood content of the ear against which the oximeter is not completely compensated. These fluctuations about the plateau create a variability in the time at which a single absolute maximum may be recorded. We have therefore previously (3) arbitrarily recorded the time to absolute maximum minus 0.5% (52 seconds average) rather than to an absolute single maximum (132 seconds average). This is justified by the calculation that there is a 54% coefficient of variation in the time to absolute single maximum saturation as opposed to the smaller and quite uniform coefficients of variation of 35%, 32% and 30%, respectively, at maximum minus 0.5%, maximum minus 1.0% and maximum minus 2.0% levels. The times to maximum minus 0.5% (average 52 seconds, S. D. 18.6 seconds) or to maximum minus 1.0% (average 36 seconds, S. D. 11.6 seconds) are more useful in the determination of abnormal delay in reaching full saturation. The mean increase upon inhalation of 100% O_2 was

3.72% with a standard deviation of 0.81% and range from 2.25% to 5.75%. Whether this signifies that the mean arterial oxygen saturation of normal persons breathing room air is 96.3% will be discussed later.

2. *The rate at which alveolar pO_2 rises during inhalation of O_2 .* The decrease in N_2 content of lung air, as it was displaced by quiet breathing of O_2 , is shown in Figure 2 with the simultaneous oximeter readings of 42 subjects. Mean values of the maximal nitrogen content of terminal expired air are plotted at 15-second intervals. The curve shows that nitrogen content decreases rapidly, *i.e.*, that oxygen content increases rapidly. For example, after the first breath of O_2 , the mean N_2 content of the terminal expired air was found to be 65%. This figure may be converted to approximate oxygen tension by the formula: $pO_2 = (100 - \%N_2 - \%CO_2) (B-47)$. Thus, assuming a content of 5.6% CO_2 , we find that the oxygen tension of the terminal expired air had a mean value of 210 mm. Hg. Similarly after 30 seconds the mean pO_2 of terminal expired air was 470 mm. Hg. The pO_2 of the terminal expired air does not accurately represent the pO_2 of "deeper" lung air as shown by the following measurements made upon the same 42 subjects with the nitrogen meter: After breathing room air, two quiet breaths of O_2 were taken. Following the second normal expiration, a further forced

maximal expiration was made. It was found that the maximal N_2 content of this "deeper" lung air had a mean value of 3.9% more than that of the air expelled at the quiet end of the same expiration. Thus the mean pO_2 of "deeper" lung air was about 28 mm. less than that of the ordinary terminal expired air. If it be assumed that this difference is due to stratification of gas in the terminal air passages, the pO_2 of the deeper lung air would still be considerably in excess of 159 mm. Hg ($210 - 28 = 182$ mm. Hg) at the end of the first breath of O_2 . However if the difference between the composition of terminal expired air and of "deeper" lung air⁴ be due to regional inequalities in lung ventilation, an explanation might be provided for the slowness of rise in oximeter readings. To test this possibility, further experiments were performed.

3. *Effect of hyperventilation upon the rate of increase in saturation.* Figure 3 shows the mean

oximeter curves of seven subjects by whom O_2 inhalation was performed both with quiet breathing and with moderate voluntary hyperventilation for the first 30 seconds. The sequence of procedures was reversed in alternate subjects. In all cases hyperventilation speeded the rate of saturation increase. With extreme hyperventilation (inhalation of O_2 beginning at the maximal expiratory position) the time to maximal saturation decreased further and approached the combined circulatory and instrumental time lag. Thus one might conclude that hypoventilation during quiet breathing is responsible for the delay and that voluntary hyperventilation will reduce it. However, vigorous breathing not only may facilitate ventilation of poorly aerated areas of the lung but also increases the pO_2 more rapidly in the well-ventilated areas, as may be inferred from the curves representing N_2 content in Figure 3.

Attempts were made to differentiate, if possible, these two factors of increased ventilation and increased pO_2 by the following method involving inhalation of 40% O_2 . 40% O_2 was chosen since its inhalation should result in an alveolar pO_2 at equilibrium well in excess of 159 mm. Hg (pO_2 of approximately 240 mm. Hg, assuming a pCO_2 of 40 mm. Hg and an R. Q. of 0.83).

Seven subjects breathed 40% O_2 for five minutes. A five-minute period of inhalation should be sufficient to reach equilibrium and overcome any delay due to hypoventilation since maximal saturations are reached in much shorter times in normal subjects. The saturation increased a mean of 2.43% and then became stable. Then without interruption of respiratory rhythm the subjects breathed 100% O_2 . In all cases a further increase in saturation occurred within one minute. The mean increase was 0.89%; the standard error of the mean was 0.091, giving a t value of 9.9, which indicates that the increase is a highly significant variation from zero. Ear thickness readings taken before and after the change to 100% O_2 were the same so that it is unlikely that a vascular change caused the alteration. However it is possible that the five-minute period with the lower diffusion pressure resulting from inhalation of 40% O_2 was not so effective in overcoming hypoventilation as was the one-minute period breathing 100% O_2 with a much higher diffusion pressure. Further experiments were done to clarify this point.

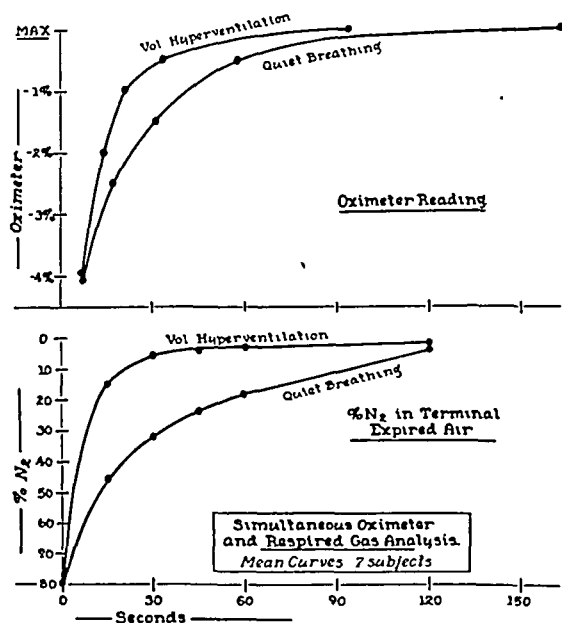


FIG. 3. THE EFFECT OF VOLUNTARY HYPERVENTILATION UPON THE RATE OF CHANGE IN ARTERIAL OXYGEN SATURATION AND CONCENTRATION OF NITROGEN IN EXPIRED GAS DURING THE INHALATION OF 99.6% OXYGEN

⁴ No one has ever analyzed the air in immediate contact with the pulmonary capillaries. Instead investigators have analyzed the various portions of lung air as they are expired chronologically during forced expiration. The terminal portion chronologically is not necessarily that air in most intimate contact with normal alveolar lining. We believe that "terminal expired air" and "deeper lung air" are both portions of so-called "alveolar air."

Seven different subjects breathed 40% O_2 for five minutes; saturation increased a mean value of 2.78% and became stable. The subjects voluntarily hyperventilated for 30 seconds and then resumed quiet breathing of 40% O_2 for two minutes. There was no evidence of a significant change; three increased by 0.5% but the others showed no change or a decrease of 0.25%. Then the subjects inhaled 100% O_2 , still breathing quietly. In all cases, a further oximeter rise occurred, the mean increase being 0.82%.

We have found that the reverse occurs when room air is breathed by a subject who had previously breathed O_2 . A subject quietly breathed 40% O_2 for five minutes. Saturation increased 3.25% above the room air value. Voluntary hyperventilation of 40% O_2 for 40 seconds, followed by quiet breathing of 40% O_2 for three minutes, produced no increase in saturation. Then 100% O_2 was breathed for three minutes; saturation increased 1%. 40% O_2 was breathed again for four minutes; saturation decreased 1%. 100% O_2 was inhaled for four minutes and saturation increased 1.25%. Saturation decreased 1% upon subsequent inhalation of 40% O_2 and a further 3% upon breathing room air.

DISCUSSION

It has been shown that complete oxygen saturation of the arterial blood is reached more slowly than expected after inhalation of O_2 . This delay (mean, 52 seconds) is considerably in excess of the lags involved in the circulation time from pulmonary to ear capillaries and in the galvanometer. Since it has been shown (11) that the uptake of O_2 by Hb can occur in a fraction of a second, this cannot explain the delay. Since the pO_2 of terminal expired air is about 210 mm. Hg and of "deeper" lung air is about 182 mm. Hg after one breath of O_2 , inadequate alveolar pO_2 should not be the explanation, unless the customary belief that a pO_2 of 159 mm. Hg will completely saturate the arterial blood is fallacious. Evidence has been presented which leads us to believe that this concept is erroneous and that alveolar and arterial tensions considerably in excess of 159 mm. Hg pO_2 must be present.

Certain questions must be considered before this conclusion can be accepted.

First, are there shunts through or around the

lungs (other than through hypoventilated alveoli) delivering sufficient venous blood into the arterial circulation that complete arterial saturation could be attained only by drawing upon O_2 dissolved by pressures greater than 159 mm. Hg? This would prolong the time to full saturation until a sufficient excess of dissolved O_2 were available. In such a case, there should be a disparity between alveolar pO_2 and arterial pO_2 . Berggren (12) found this difference to be only 11 mm. Hg and Fasciolo and Chiodi (13) estimated it as 35.8 mm. Hg after some minutes of O_2 inhalation. This represents a venous admixture of 0.6–2.0% of the total blood flow assuming a normal A–V difference. However, in our subjects breathing 40% O_2 , it may be calculated that there can be enough dissolved O_2 to saturate completely a venous admixture of 4% of the total blood flow, without dropping the pO_2 below 160 mm. Hg. Therefore, if a pO_2 of 160 mm. Hg were sufficient to completely saturate hemoglobin, the inhalation of 40% O_2 should saturate arterial blood completely despite the presence of two to six times the amount of venous admixture thought to occur in man. Consequently the additional increase in saturation that occurs on breathing 100% O_2 following 40% O_2 can scarcely be explained solely on the basis of further saturation of venous shunted blood.

A second question is whether the blood in the vasodilated ear represents true arterial blood or whether ear metabolism creates an unsaturation that can be overcome only by very high oxygen tensions. Lilienthal and Riley found no significant difference between the O_2 saturation of arterial blood and cutaneous blood obtained from the ear warmed by radiant heat (14). Comroe and Walker (15) found that the average oximeter rise when O_2 inhalation followed room air was 3.8% while the saturation rise determined simultaneously by direct determinations upon arterial blood amounted to only 2.5%. This might mean that the oximeter scale is imperfectly calibrated in the 96–100 range or that the saturation of the ear blood analyzed by the oximeter is 1.3% less than arterial blood. Even if the latter is true, an initial arterial pO_2 of 240 mm. Hg should provide sufficient excess dissolved O_2 to saturate this without reducing final arterial pO_2 below 159 mm. Hg. Since the inhalation of 100% O_2 leads to an alveolar pO_2 in excess of this by the second breath,

even the maximal unsaturation credited to ear blood should be overcome very promptly. Thus this factor alone cannot explain the slower rise of O_2 saturation to its maximum. This assumes that there is little or no difference between alveolar and arterial pO_2 during the period of rapid change in alveolar gas tensions; this remains to be proven experimentally, though preliminary experiments indicate that this assumption is valid.

It appears that no one of the factors of delayed ventilation, venous shunts other than through poorly ventilated alveoli, or ear metabolism will completely explain the slow rise in saturation upon O_2 breathing, though each may occur. We believe that a more important factor is the failure of complete saturation of Hb to occur at an arterial or alveolar pO_2 of 160 mm. Hg. The extreme upper end of the oxygen dissociation curve of blood hemoglobin has not been so well defined as other parts of the curve, principally due to the small changes in saturation with large changes in oxygen tension, and to the technical limitations of the methods used. Adair (16) proposed that the hemoglobin molecule, which is probably a four-unit aggregate, takes up 4 O_2 molecules in successive steps, each reaction involving the addition of one O_2 molecule having a different equilibrium constant. Ferry and Green (17) also found that their data on the uptake of O_2 by Hb could be represented by a 4 constant equation, that is, that Hb appears to behave as though it were a mixture of substances, the reactions of each obeying the general form of the mass law equation. It has been suggested by Dr. F. J. W. Roughton (18) that in the very high saturation range considered here, we are dealing with only the last one of these equilibrium reactions, in which unsaturation is inversely proportional to the pO_2 . It must follow therefore that, if blood hemoglobin is 97% or 98% saturated at an oxygen tension of 100 mm. Hg, and if the same equilibrium constant holds for increasing tension-saturation relationships, 100% saturation cannot be attained at a pO_2 of 160 mm. Hg. Moreover such a relationship would be graphically expressed by a reciprocal curve. If we look at Figures 1 and 3 and visualize increasing O_2 tension plotted on the time axis, the oximeter curves (saturation) definitely suggest such a reciprocal relationship. The manner in which saturation decreases when room air is breathed

following oxygen inhalation, as shown in Figure 4, supports this view. By comparison with Figure 1, it is apparent that the fall in saturation follows a much different time course than does the rise. However, when O_2 tension is being reduced in the manner shown in Figure 4, saturation should decrease in a course approximately like the observed fall, if following the reciprocal relationship.

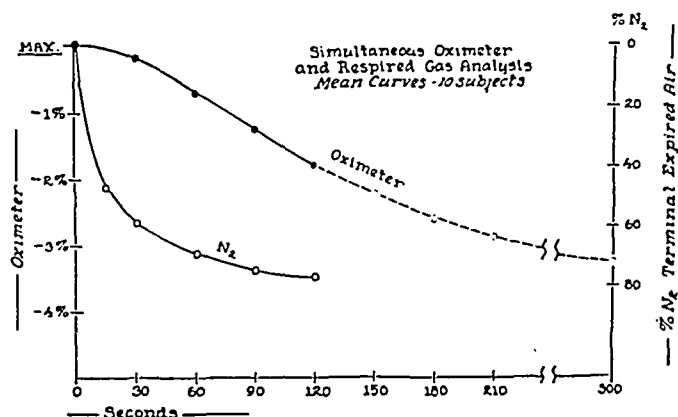


FIG. 4. SIMULTANEOUSLY RECORDED CHANGES IN ARTERIAL OXYGEN SATURATION (OXIMETER) AND CONCENTRATION OF NITROGEN IN EXPIRED AIR DURING THE CHANGE FROM INHALATION OF OXYGEN TO ROOM AIR

In view of the previously noted multiplicity of variables which affect the final oximeter readings, it is doubtful whether oximeter data obtained in this way, which is in essence a rapidly changing series of equilibrations, should be interpreted more rigidly in an attempt to define the upper end of the oxygen dissociation curve.

A final question which we cannot evaluate lies in the possibility of gradual reversion to oxyhemoglobin of previously inactive forms of hemoglobin.

The rate of increase in arterial oxygen saturation following the inhalation of oxygen is the end result of various processes such as (1) movement of oxygen to the alveoli, which involves respiratory rate, depth and mixing of gases in the lungs; (2) diffusion of O_2 across the alveolar-capillary membrane into the red cells; (3) conversion of reduced Hb to HbO_2 ; and (4) transport of HbO_2 to the site of measurement. As one or more of these various functions are disturbed by diseases, one might expect the disturbance to be revealed in such a measurement. Figure 5 illustrates curves from a patient with emphysema and from

another with primary pulmonary arteriolar sclerosis (Ayerza's disease). It is seen that one can measure (a) the delay time (mouth to lung to ear time), (b) the total rise in oximeter reading, and (c) the rate of rise of oxygen saturation. We believe that this test is useful as a simple, innocuous, objective, overall measurement of certain aspects of lung function. It is not an analytical test but is useful as a screening test preliminary to a more complete study of cardiopulmonary function.

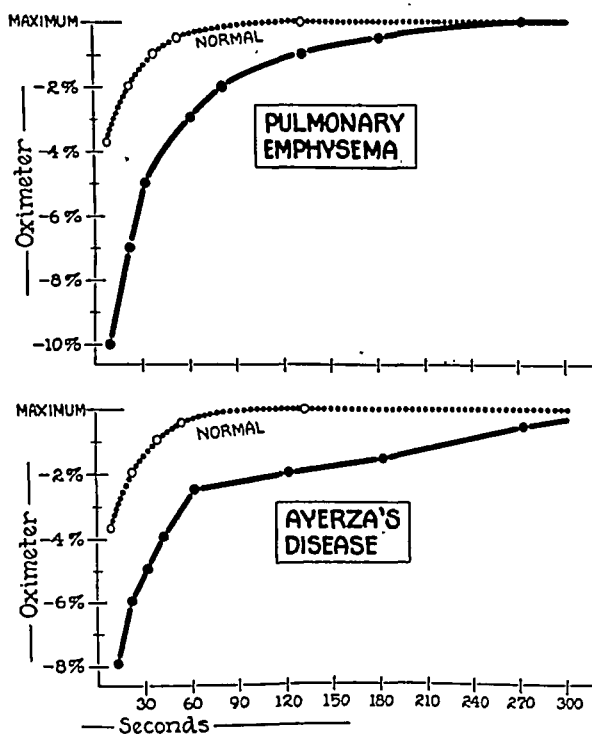


FIG. 5. RATE OF INCREASE OF ARTERIAL OXYGEN SATURATION IN A PATIENT WITH PULMONARY EMPHYSEMA (UPPER) AND IN A PATIENT WITH PULMONARY ARTERIOLOSCLEROSIS (LOWER)

SUMMARY

1. Measurements were made with the oximeter of: a. The increase in arterial oxygen saturation which follows quiet and vigorous breathing of 99.6% oxygen. b. The decrease in arterial oxygen saturation which occurs when room air is breathed following a period of oxygen inhalation. c. The increase in arterial oxygen saturation which occurs when 99.6% oxygen is breathed following a period of 40% O_2 inhalation.

2. Continuous measurements of the N_2 content of the respired gases were made simultaneously with oximeter recordings.

3. The attainment of maximum arterial saturation following inhalation of 99.6% oxygen requires a longer time than is readily explainable, if uniform lung ventilation and complete saturation of Hb at an arterial pO_2 of 160 mm. Hg are assumed. Non-uniformity of the composition of alveolar air was demonstrated; but the magnitude of this factor is not believed to be sufficient to explain the delay in attaining maximum saturation. Neither the shunting of venous blood into the arterial circulation, nor the metabolic reduction of oxyhemoglobin in the ear adequately explains the delay. The occurrence of a small increase in oxygen saturation of the arterial blood at arterial oxygen tensions between 160 and 670 mm. Hg will explain the experimental results.

4. The usefulness of the oximeter rate as a simple, preliminary screening test for the detection of pulmonary disability is discussed.⁵

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⁵ The authors wish to express their appreciation to Dr. John Lilly for his advice on the operation of the nitrogen meter and to Dr. Edwin Cornish, Stella Botelho, and Sylvia Himmelfarb for their assistance.

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THE EFFECT OF SPINAL ANESTHESIA ON THE RENAL ISCHEMIA IN CONGESTIVE HEART FAILURE¹

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One of the effects of a decreased cardiac output in chronic congestive heart failure is a disproportionate decrease in the renal blood flow. We have calculated the renal fraction of the cardiac output to be reduced to about two-fifths of normal and have shown that there is marked efferent arteriolar constriction with increased intraglomerular filtration pressure in congestive heart failure (1). Although Merrill and co-workers (2) have demonstrated that renin is present in increased amounts in the renal venous blood of some patients with congestive failure, we wondered if renal vasoconstriction might also result from neurogenic stimulation when the cardiac output falls; in which case, hyperemia should be produced by blocking the reflex pathways. In the present study the interruption of the autonomic vasoconstrictor pathways was effected by means of high spinal anesthesia. As will be seen from the results obtained in these experiments, renal hyperemia does not occur in response to the abolition of sympathetic vasoconstrictor impulses.

METHODS

Fourteen patients with varying degrees of congestive heart failure were the subjects. They were examined in the post-absorptive state employing the clearance of mannitol (or sodium thiosulfate in two instances) as a measure of glomerular filtration rate, and the clearance of p-aminohippuric acid³ as a measure of effective renal plasma flow as described in a previous report (1).

After three or more control clearance periods were obtained, 48 mg. of ephedrine sulfate were injected intramuscularly in order to counteract any fall in arterial pressure, consequent to the high spinal anesthesia. The patient was then placed in the lateral position and the subarachnoid space entered between the levels of L 3

and L 4. Metycaine d,l-3-Benzoxo-1-(2-methylpiperidino) propane Hydrochloride (120-150 mg.) mixed with equal parts of spinal fluid was the anesthetic agent. Procaine was not used because it has an amino group in the para position of the benzene nucleus and would result in falsely high values for p-aminohippurate (3). Moreover, a longer acting anesthetic agent was desirable. After the metycaine was injected rapidly, the patient was placed in the prone position with the pelvis tilted at an angle of -10 to -15 degrees. This position was maintained for about 10 to 20 minutes after which the patient was turned flat on his back. In two instances, because of the patient's severe dyspnea and orthopnea, the lumbar puncture was made in the upright position. In two other cases, because of unsatisfactory ascent of the anesthesia, a second injection of metycaine was necessary before the test periods were started. The test clearance periods were started after the highest level of sensory anesthesia was reached which was usually between 25 and 40 minutes after spinal injection. In several instances when the anesthesia reached the first thoracic segment and there was almost complete intercostal paralysis, oxygen was administered. Artificial respiration was available, but was never required. Blood pressure was taken by auscultation of the brachial artery at frequent intervals during the control and anesthesia periods. The figures given in Table I are the means of several readings in each period.

RESULTS

The maximal level of anesthesia was unsatisfactory in three patients, who consequently have been omitted from consideration. The data of the remaining eleven are summarized in Table I. It will be seen that the lowest level of anesthesia reached was the sixth thoracic segment. Smith and associates (4) have shown that with this degree of sensory anesthesia there is abolition of the reflex vasoconstrictor responses to anoxia, hypercapnia and tilting. We believe, therefore, had there been any reflex vasoconstrictor impulses reaching the kidney they would have been effectively blocked.

As to the blood pressure changes in these patients, the only generalization that can be made is that the variations tended to be minimal. We were able to utilize the action of ephedrine in maintain-

¹ This study was aided by a grant from the United States Public Health Service and the Martha M. Hall Foundation.

² Martha M. Hall Foundation Fellow in Medicine.

³ Liberal quantities of mannitol and p-aminohippuric acid were supplied by the Medical Research Division of Sharp and Dohme, Inc. Sodium thiosulfate was supplied by Winthrop and Co.

TABLE I

*Effects of spinal anesthesia on renal circulation in congestive heart failure **

Patient	Sex	Age	Diagnosis†	Maximal level of anesthesia	Glomerular filtration rate cc. per min.			Renal plasma flow cc. per min.			Filtration fraction per cent			Blood pressure mm. Hg		Remarks
					Control	Anesthesia	Per cent change	Control	Anesthesia	Per cent change	Control	Anesthesia	Per cent change	Control	Anesthesia	
1. C. R.	F	36	RHD	T 6	48	38	-21	88	75	-15	55	51	-7	110/84	78/60	Ephedrine omitted
2. I. F.	F	43	HHD and RHD	T 2	60	62	+3	210	198	-6	29	31	+7	164/110	190/104	
3. A. M.	F	16	Cong. HD	T 2	77§	69	-10	127	118	-7	61	58	-5	166/90	170/106	No edema. Compensation restored
4. C. S.	M	60	ASHD	T 1	85	72	-15	234	206	-12	36	35	-3	108/60	98/60-70	
5. B. M.	F	50	RHD	T 4	91§	79	-13	280	290	+4	33	27	-18	110/74	106/70	
6. E. Mc.	M	20	RHD	T 1	56	76	+36	137	170	+24	41	45	+10	150/45	130/40	Pyrogen reaction. Temp. rise to 103° Minimal edema. Compensation restored
7. J. S.	F	35	RHD	T 6	73	72	-1	243	236	-3	30	31	+3	110/80	106/74	
8. S. B.	M	39	RHD	T 1	33	31	+6	116	171	+47†	28	18	-36	130/90	110/70	
9. E. S.	M	38	HHD	T 3	99	118	+19	307	373	+21	32	32	0	185/125	208/128	
10. F. I.	M	43	ASHD	T 5	61	55	-10	145	143	-1	42	39	-7	96/80	90/74	
11. J. L.	M	39	ASHD	T 3	84	93	+11	213	229	+8	39	41	+5	98/80	96/70	
Mean change							0			+1			-2			

* All values are averages of three or more periods.

† Cong. HD, RHD, ASHD, HHD = Congenital, rheumatic, arteriosclerotic and hypertensive heart disease, respectively.

‡ This figure was omitted in calculating mean change, due to interfering pyrogen reaction.

§ Sodium thiosulfate clearance used as a measure of glomerular filtration rate.

|| Determined as the highest level of absolute loss of sensation to pin prick.

ing the systolic pressure during high spinal anesthesia. In the one case (C. R.) in which it was omitted there was a moderate drop in the mean pressure. Ephedrine sulfate in the amounts used in these patients has been shown to have no effect on the renal circulation (5). We have confirmed this in control observations on two patients with severe congestive failure. Therefore, with only a single exception (C. R.), any changes in the renal circulation which were obtained, were not due to a decrease in driving force. Similarly, the use of ephedrine precluded the possibility that any renal hyperemia which might occur would be offset by the fall in mean pressure during anesthesia.

Except for the subjects E. Mc. and S. B., there was no significant change in either glomerular filtration rate or renal plasma flow in any of the patients. E. Mc. (Figure 1) had an increase in plasma flow of 24 per cent and an increase in glomerular filtration rate of 36 per cent in spite of a slight fall in systolic pressure. We have no adequate explanation for the unusual changes observed. C. R. had a decrease in all the functions studied which we attribute largely to the drop in mean pressure. Figure 2 (I. F.) illustrates the

typical response seen in the majority of these patients. Taking the series as a whole, the mean change in the renal circulation is nil.

Patient S. B. developed a pyrogen reaction immediately after the spinal anesthesia. It will be seen that the renal plasma flow was increased by 47 per cent, the glomerular filtration rate was unchanged and the filtration fraction decreased to normal values. This is interpreted as being due to efferent arteriolar dilatation (6), and indicates that neurogenic control of the renal vessels is not required for its completion. The pyrogen reaction with its renal circulatory changes also occurs in the sympathectomized hypertensive patient (7).

It is of some interest to note that several of the patients who were dyspneic and orthopneic before spinal anesthesia could lie flat with no apparent discomfort while the clearance studies under anesthesia were completed. Apparently, the marked diminution of afferent impulses from atonic limbs and abdominal muscles, the decreased venous pressure secondary to venous stagnation with resulting decrease in reflex respiratory stimulation (8), produced the desired effects.

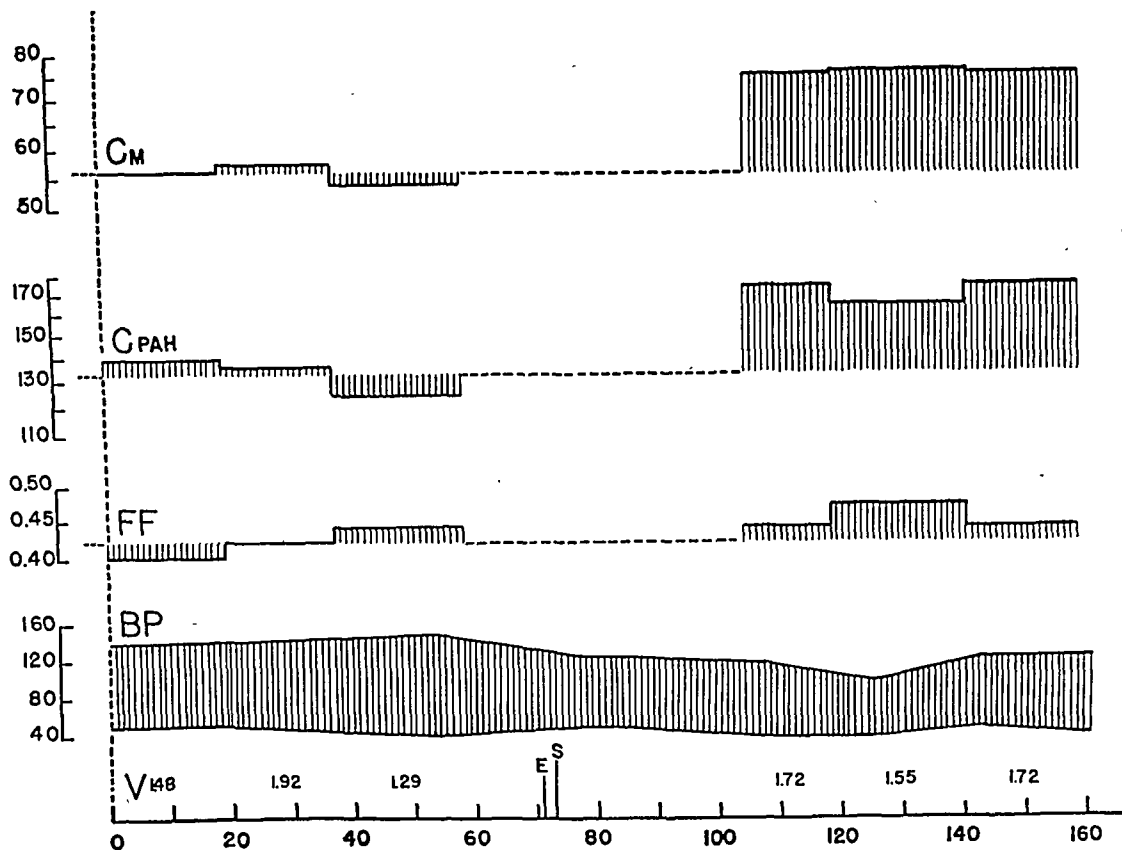


FIG. 1. THE UNUSUAL RESPONSE OF THE RENAL CIRCULATION TO SPINAL ANESTHESIA IN PATIENT E. MC. WITH SEVERE CONGESTIVE FAILURE

This patient had an increase in renal plasma flow and glomerular filtration rate of 24 and 36 per cent, respectively, during sensory anesthesia up to the first thoracic segment as compared to control periods before anesthesia. This occurred in spite of a drop in systolic pressure.

C_M = Mannitol clearance = glomerular filtration rate (cc. per minute).

C_{PAH} = P-aminohippurate clearance = effective renal plasma flow (cc. per minute).

FF = Filtration fraction (fraction of the renal plasma flow filtered at the glomerulus).

BP = Auscultatory blood pressure in mm. Hg.

V = Urine flow (cc. per minute).

E = Time of ephedrine sulfate (48 mg.) injection.

S = Time of spinal metycaïne injection.

COMMENTS

Our results indicate that the renal ischemia with marked efferent arteriolar constriction and the concomitant reduction in the renal fraction of the cardiac output so regularly observed in established congestive failure, are not maintained by sympathetic activity. That reflex vasoconstriction with shunting of blood from the kidney may operate in acute heart failure is not denied by these studies. Neurogenic stimuli do affect the renal vascular bed. Smith (5) has shown that orthostasis and emotional stimuli induce renal ischemia. An in-

crease in renal vasoconstriction with further depression of renal plasma flow is also obtained in essential hypertension (9) and in congestive failure (10) under these conditions. In some patients with hypertension it appears that neurogenic factors are the *modus operandi* of the renal ischemia. Page, Corcoran and co-workers (11) have observed renal hyperemia under spinal anesthesia in a group of "neurogenic" hypertensives. However, in some of their patients and in the reports of other workers (6, 12), no such decrease in renal arteriolar resistance occurs. The use of tetra-

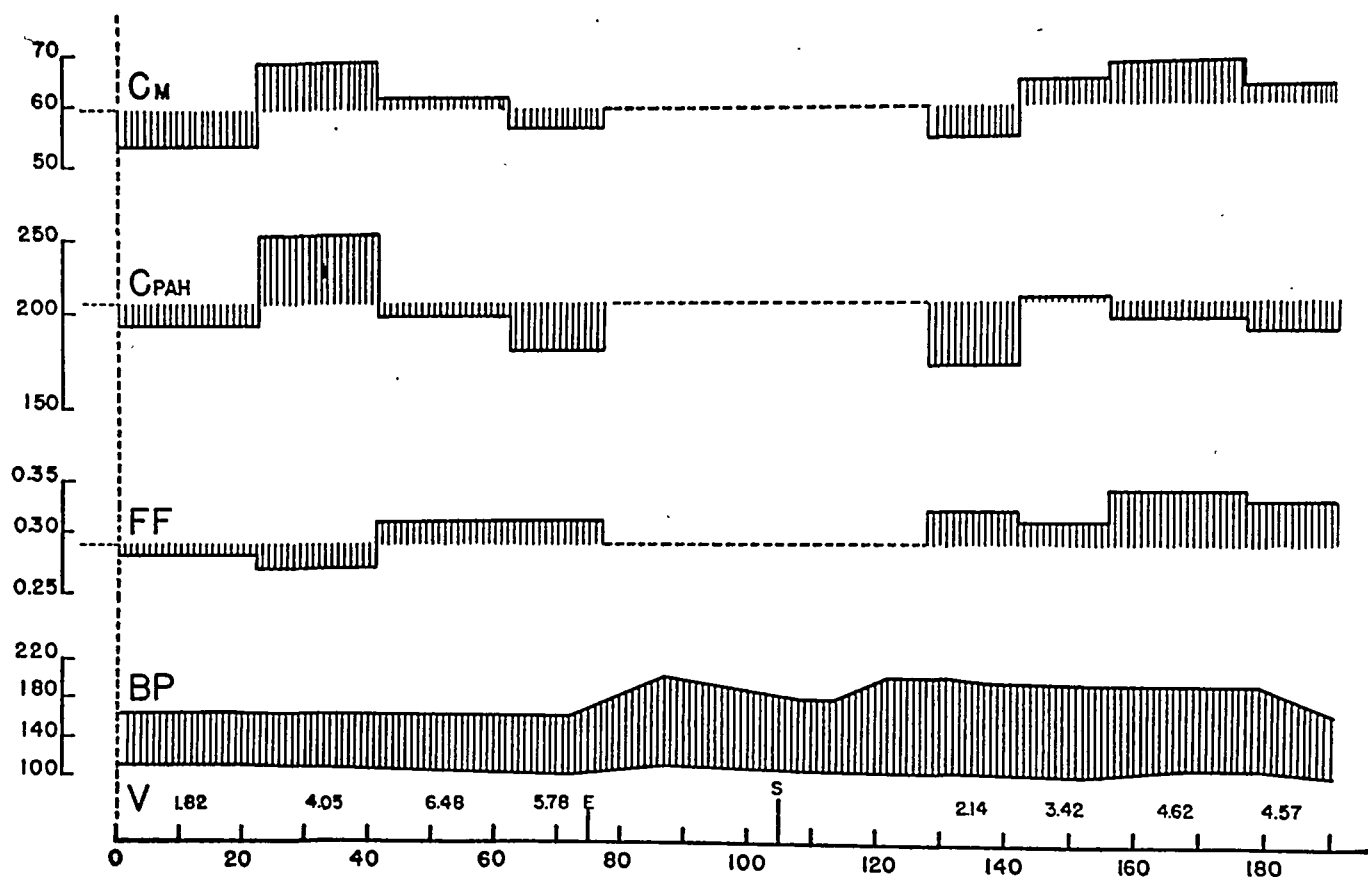


FIG. 2. THE TYPICAL RESPONSE OF THE RENAL CIRCULATION IN CONGESTIVE HEART FAILURE TO HIGH SPINAL ANESTHESIA. PATIENT I. F. SYMBOLS AS IN FIG. 1

There is no significant change in the renal plasma flow or glomerular filtration rate during anesthesia to the second thoracic segment as compared to control periods before anesthesia.

ethylammonium ion as an autonomic ganglionic blocking agent by Lyons and co-workers (13) was reported to have produced renal vasodilatation; the latter, however, consisted merely of a maintained renal blood flow in the presence of a fall in blood pressure. No renal hyperemia was observed. It would be of interest to follow the renal circulatory changes with this drug if the concomitant drop in blood pressure could be avoided.

Our inability to alter renal blood flow in chronic heart failure by removal of nerve impulses makes it highly improbable that the renal changes associated with congestive failure are produced by this selfsame mechanism. It would therefore appear far more likely that the renal circulatory changes observed, are mediated through some humoral mechanism. This mechanism must involve the formation of a substance or substances either local in the kidney or extrarenal and would account for the observed phenomena in heart failure; namely, the increased peripheral resistance with

maintenance of a normal blood pressure in the presence of a decreased cardiac output and the high filtration fraction in the presence of a decreased renal blood flow. Merrill (2) has demonstrated increased amounts of renin in the renal venous blood in eight of 11 patients with congestive heart failure. Shorr, Zweifach and associates (14) have reported the release of vaso-excitatory material (VEM) from ischemic kidneys *in vivo* and its production anaerobically by kidney cortex *in vitro*. May it not be liberated from the kidneys in chronic heart failure as a result of the renal hypoxia? This is now being investigated.

SUMMARY

1. Renal plasma flow and glomerular filtration rates were determined in 11 patients with chronic congestive heart failure before and after high spinal anesthesia. The mean change in these renal functions was insignificant.

2. It is concluded that a neurogenic mechanism is not required for the maintenance of the increased tone of the renal arterioles in chronic congestive heart failure.

3. The nature of the humoral mechanism involved is unknown, but among the possibilities are renin and (VEM), both released in response to renal ischemia.

ACKNOWLEDGMENTS

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THE CHANGES IN THE SERUM PROTEINS IN PATIENTS WITH EXPERIMENTALLY INDUCED INFECTIOUS HEPATITIS¹

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The alterations in the partition of the serum proteins of patients with infectious hepatitis have been described as an early decrease in serum albumin with an increase in serum globulins (1-4). Both alpha and beta globulins are augmented but the greatest increase is in the gamma globulin fraction. It has been pointed out that qualitatively similar changes occur in several other acute infectious diseases, and it has been suggested that these alterations are *non-specific* in character (5). Such changes have been described as occurring in malaria (6), pneumonia (7), scarlet fever (5), rheumatic fever (5), typhus (8, 9), and infectious mononucleosis (10).

Many of the early studies were made by the Howe method of salt fractionation (11), although several more recent descriptions of electrophoretic analyses have supplemented these studies and have defined more accurately the changes in the partition of serum proteins. The discrepancy between the results of analysis of pathologic sera by electrophoresis and the Howe method of salt fractionation has made the former the method of choice, although it is not applicable to clinical use (12).

Recently, another method of salt fractionation of serum proteins by differential precipitation with 19.6 per cent and 26.8 per cent sodium sulfate has been described (13). Excellent correlation of the results of this method and electrophoretic analysis of both normal and pathologic sera has been reported (14, 15).

During the past three years, experiments conducted by the Neurotropic Virus Disease Commission on the transmission of infectious hepatitis to human volunteers have made it possible to study the pattern of change in the serum proteins of such patients throughout the course of disease. It is the purpose of this paper to report the al-

terations in serum proteins, as determined by a method of differential precipitation, in 29 patients during the course of experimentally induced infectious hepatitis.

METHODS AND MATERIALS

Subjects. The subjects were previously healthy, male human volunteers, ranging in age from 19 to 29 years. These men contracted infectious hepatitis experimentally after inoculation or ingestion of material known to contain hepatitis virus. The diagnosis of infectious hepatitis was made on the basis of characteristic symptoms and signs accompanied by fever and consistent deflection of the bromsulfalein dye² retention (16) and cephalin-cholesterol flocculation (17) tests. All patients in this report had clinical jaundice. Two of the patients included in this series had bacteremia with *Sal. cholera suis* superimposed on their experimentally induced infectious hepatitis (18). The changes in serum proteins in these two patients were qualitatively similar to the rest of the group, although one of these men had more extreme deflections associated with his more severe illness.

Virus. The strain of virus used in this laboratory was originally obtained from the stool of a U. S. Army soldier who contracted *epidemic infectious hepatitis* in Sicily in 1943 (19). It has been through four passages in human volunteers to date. This agent is filtrable through a Seitz EK filter, and withstands heating to 56° C. for at least 30 minutes (20). It has produced the disease in 29 out of 40 human volunteers after parenteral or oral inoculation, with incubation periods ranging from 15 to 34 days.

Laboratory observations. 235 determinations of total serum protein, serum albumin, euglobulin and pseudoglobulin were made on the 29 human volunteers. Total nitrogen was determined, by the micro-Kjeldahl technique, in the whole serum and on aliquots of the various fractions following differential precipitation. All analyses were performed in duplicate. The non-protein nitrogen was determined by the method of Bock and Benedict (21), using a Pregl steam-distillation apparatus. Fractionation of serum proteins was performed by precipitation with 26.8 per cent and 19.6 per cent sodium sulfate, according to the method of Majoor (13) modified by Milne (14): The sera used had been stored at dry

¹ This investigation was conducted in part with the aid of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

² Retention of 10 per cent of dye 30 minutes after the intravenous injection of 5 mgm. of bromsulfalein per kgm. of body weight was considered the maximum normal.

ice-box temperature for periods ranging from one to two and a half years. The range and average determinations of the various components of the serum proteins of these 29 human volunteers were compiled before experimental inoculation. The results appear in Table I, and are used as averages against which the deflections of individual responses during the course of disease are plotted in various charts.

In these experiments, the total serum proteins and their components were determined once every ten days for one month after the onset of disease; at least once every two

TABLE I

Range and average amounts of components of serum protein in 29 healthy human volunteers before experimental inoculation with virus of infectious hepatitis

	Range gm./100 ml.	Average
Total protein	5.60-8.36	6.93
Albumin	3.58-4.99	4.35
Globulin	2.00-3.57	2.57
Euglobulin	1.04-2.40	1.75
Pseudoglobulin	0.60-1.17	0.81

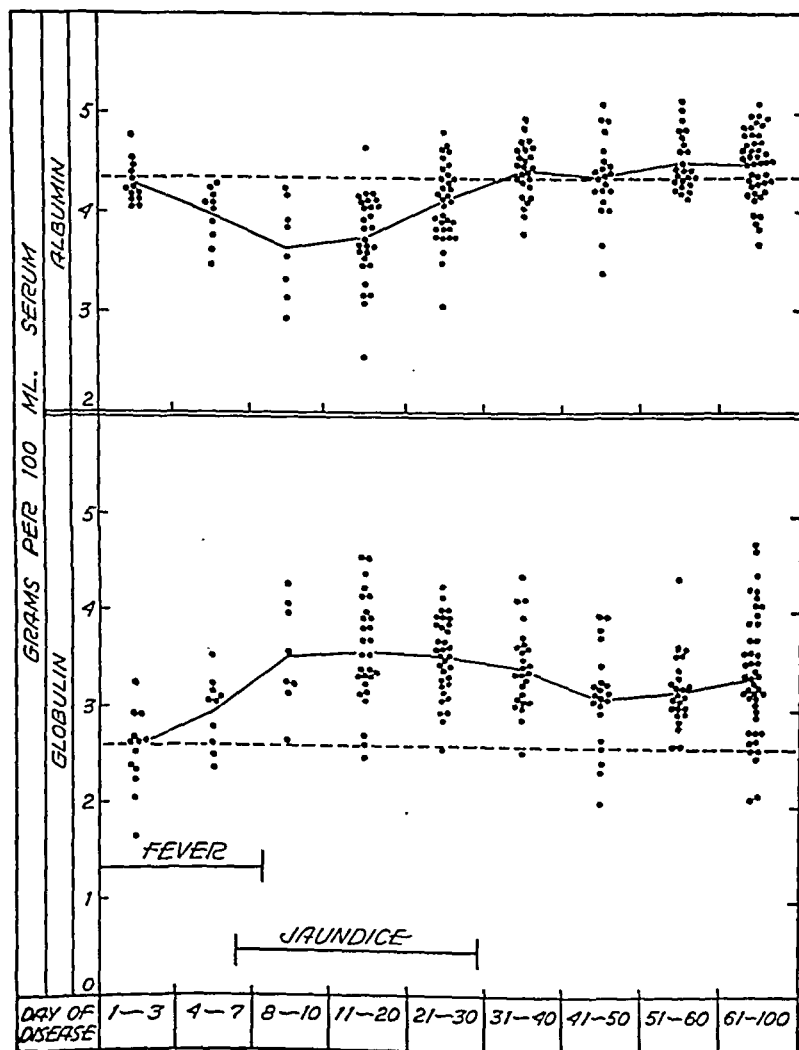


FIG. 1. DETERMINATIONS OF ALBUMIN AND GLOBULIN IN THE SERUM OF 29 PATIENTS WITH EXPERIMENTALLY INDUCED INFECTIOUS HEPATITIS

Black dots indicate the individual determinations which are expressed as an average by the unbroken lines. The duration of fever and jaundice and the appearance time of jaundice represent the averaged experiences of the 29 patients. The horizontal (broken) lines indicate the average of the pre-inoculation determinations in the 29 patients.

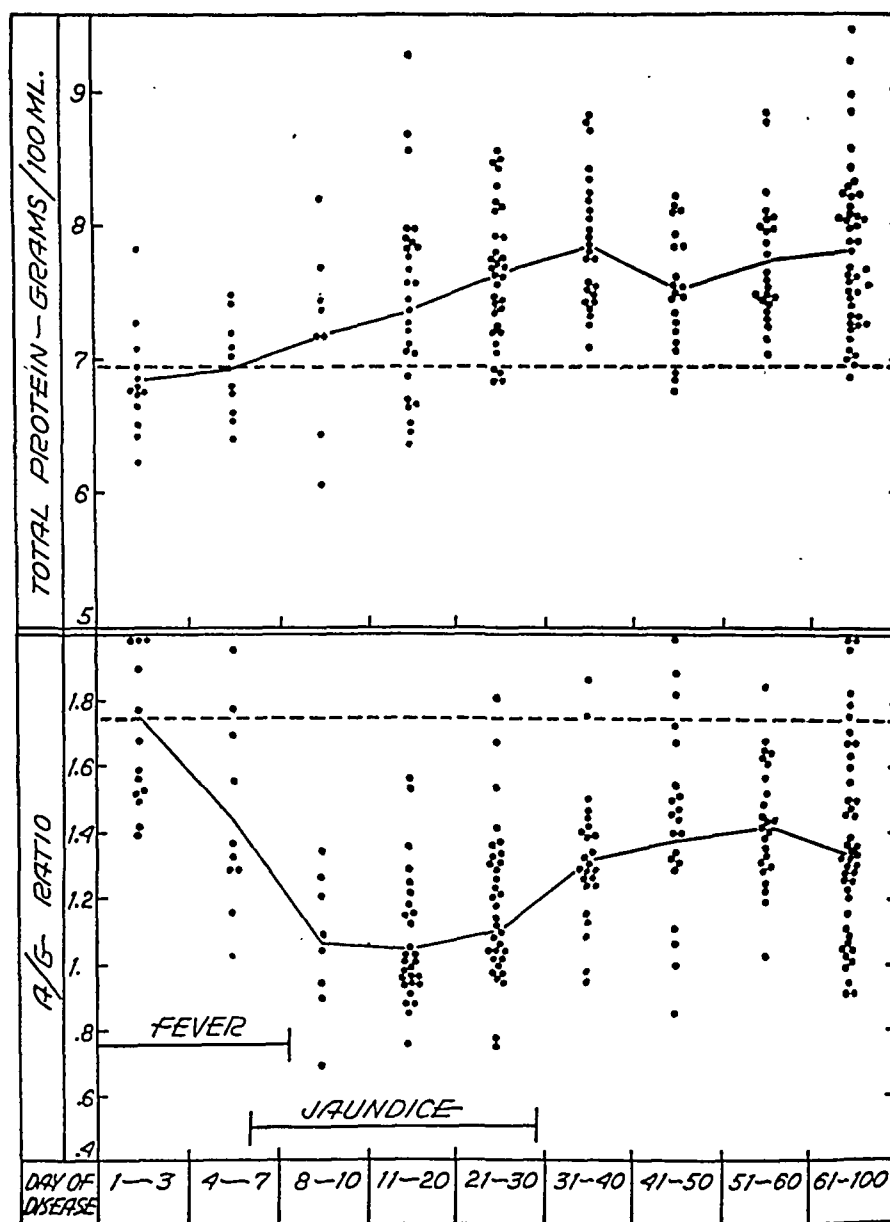


FIG. 2. TOTAL SERUM PROTEIN AND ALBUMIN/GLOBULIN RATIOS IN 29 PATIENTS WITH EXPERIMENTALLY INDUCED INFECTIOUS HEPATITIS

Black dots indicate the individual determinations which are expressed as an average by the unbroken lines. The duration of fever and jaundice and the appearance time of jaundice represent the averaged experiences of the 29 patients. The horizontal (broken) lines indicate the average of the pre-inoculation determinations in the 29 patients.

weeks for the second month after the onset; and at least once in the third month.

RESULTS

When determinations of the total serum proteins and their components were made this frequently, a regular pattern of response in infectious hepatitis was observed. The 29 patients reported here may be almost equally divided into

two groups. One group had an acute onset of disease with fever. The other group had an insidious onset with vague abdominal symptoms for periods of two to 11 days before the appearance of fever.

By using the appearance of fever as a fixed point, it is possible to present the alterations occurring in the serum proteins in all 29 patients of this series. When this is done, and the average

duration of fever and jaundice and the appearance time of jaundice are recorded, it is seen that moderate alterations in the serum proteins occur in a certain proportion of patients during the febrile phase before the appearance of jaundice, and that with defervescence and appearance of jaundice at the beginning of the second week almost all patients tested showed considerable deflection from normal (Figure 1).

For purposes of convenience, the results of the determinations of serum proteins and their components are described as occurring in three phases; namely (1) the febrile (pre-icteric) phase, (2) the icteric (post-febrile) phase, and (3) the convalescent (post-icteric) phase.

Febrile (pre-icteric) phase. The duration of fever ranged from four to 14 days, averaging eight days. During the first week after the appearance of fever, there is little or no change in the total proteins, but there is a mild decrease in serum albumin and an increase in serum globulin. These slight changes become more evident after the third day.

Icteric (post-febrile) phase. Clinical jaundice appeared from three to 12 days after the beginning of fever, averaging six days. The duration of jaundice ranged from eight to 31 days, with an average of 20 days. Characteristically, clinical jaundice appears with defervescence at the end of the first week. Early in the second week, there is a sharp decrease in amount of serum albumin accompanied by a proportionally greater increase of serum globulin, to the extent that the total amount of serum protein increases and the albumin/globulin ratio is reversed (Figure 2). The albumin may diminish to as little as 2.5 gm. per 100 ml., and the globulin increase to 5.0 gm. per 100 ml. serum. The increment in globulin consists largely of euglobulin, although the pseudoglobulin increases slightly so that there is considerable alteration of the euglobulin/pseudoglobulin ratio (Figure 3). During the third week of disease, the serum globulin rises still further, accompanied by a slight increase in serum albumin, so that the total amount of serum protein increases. Through the fourth week, as jaundice wanes, the serum albumin continues to rise, while the serum globulin remains constant. The total protein increases slightly, reflecting the increase in serum albumin. During the third week, the euglobulin continues

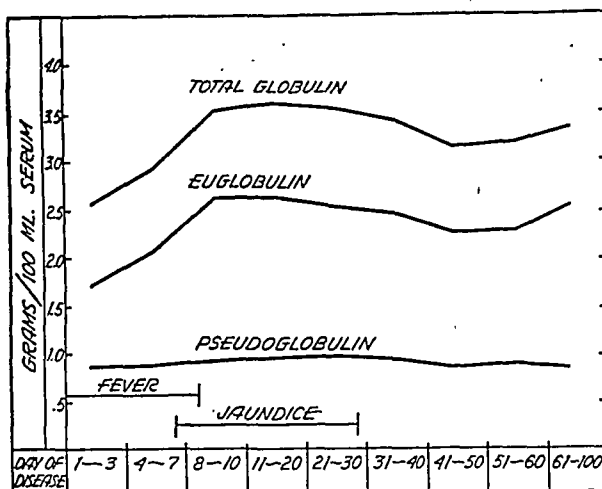


FIG. 3. AVERAGED DETERMINATIONS OF THE TOTAL GLOBULIN, EUGLOBULIN AND PSEUDOGLOBULIN IN THE SERUM OF 29 PATIENTS WITH EXPERIMENTALLY INDUCED INFECTIOUS HEPATITIS

The duration of fever and jaundice and the appearance time of jaundice represent the averaged experiences of the 29 patients.

to increase, declining slightly in the fourth week, while the pseudoglobulin rises gradually during this period, reaching a peak in the fourth week.

Convalescent (post-icteric) phase. During the fifth week of disease, the amount of serum albumin increases to a point slightly above normal, continuing at this level during the next three weeks. The serum globulin slowly declines during this period, becoming normal in an occasional person although without reaching the average normal level for the group. The euglobulin and pseudoglobulin diminish proportionately. From the eighth to the 14th week, the serum albumin continues at just above a normal level with occasional exceptions, and the serum globulin remains somewhat elevated, resulting in a slight increase in total protein during this period, with an albumin/globulin ratio below normal. The amount of serum albumin was found to be below 4 gm. per 100 ml. during this period in two patients with relapse, and in one patient with a prolonged convalescence.

DISCUSSION

The data presented here indicate that a regular pattern of alteration in the serum proteins occurs during the course of infectious hepatitis, *experimentally induced*, in human volunteers. This is

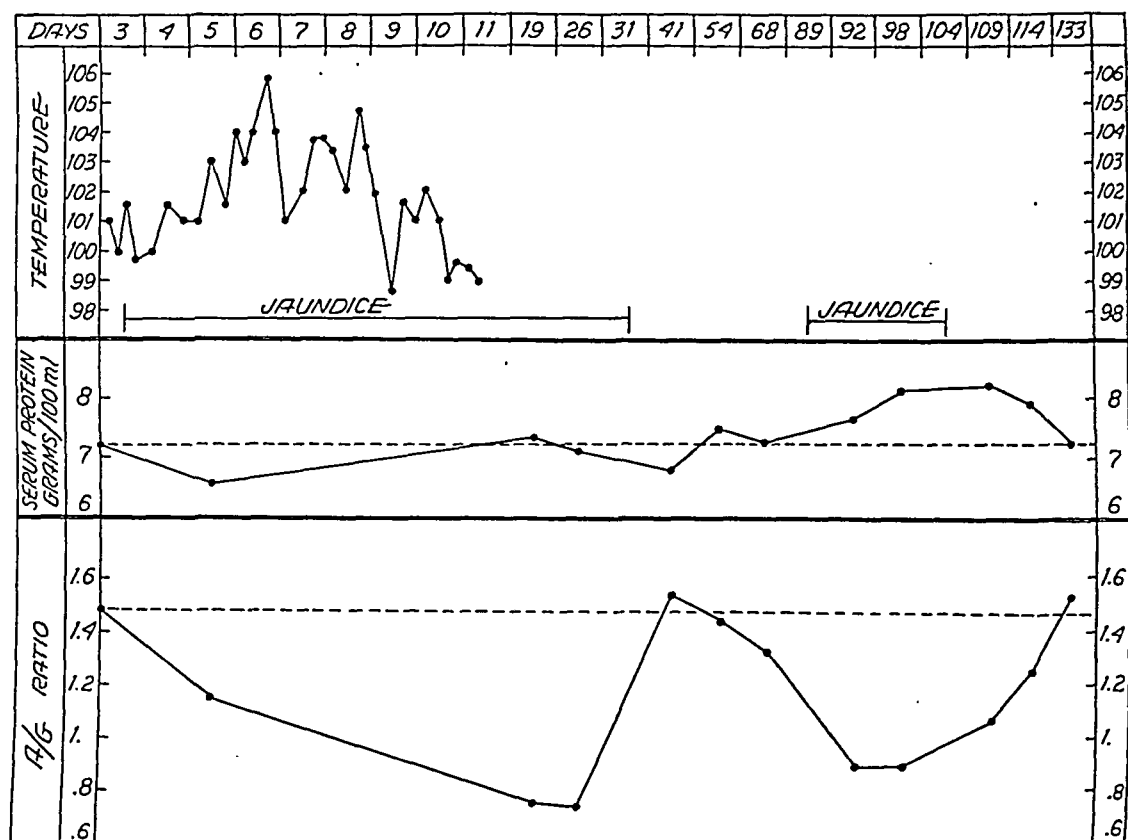


FIG. 4. COURSE OF DISEASE WITH RELAPSE IN PATIENT WITH EXPERIMENTALLY INDUCED INFECTIOUS HEPATITIS

Rectal temperatures are recorded. The horizontal (broken) lines indicate the pre-inoculation determinations of total serum protein and albumin/globulin ratio.

in general agreement with the findings of others (1-4) working with both the naturally occurring and experimentally induced disease; it is also similar to the response observed in a number of other acute infectious diseases of diverse etiology.

It is of interest to note that although the serum albumin reaches essentially normal limits by the fifth week after onset, the amount of serum globulins remained elevated well above normal in almost all patients for as long as three and a half months. The exact significance of this finding is undetermined. The increase of globulin is almost wholly in the euglobulin fraction late in convalescence. Since this fraction has been identified with the beta and gamma globulin fractions separated by electrophoresis, the possibility must be considered that a portion of the increment is related to persistence of antibody.

The degree and duration of depression of serum albumin are in direct proportion to the severity of the hepatitis. In two patients who suffered relapse during an apparently normal convales-

cence, the characteristic pattern of alteration in serum proteins was repeated at this time, with depression of serum albumin and increase in globulins (Figure 4). Similar findings have been recorded in malaria and rheumatic fever (5, 6).

SUMMARY

1. The results of serial observations of the alterations in serum proteins of patients during *experimentally induced* infectious hepatitis are presented.

2. Although minor changes may occur in the first two or three days of fever, the characteristic response is associated primarily with the period of defervescence at the end of the first week.

3. The pattern of this response is characterized by a sharp decrease in serum albumin with an increase in serum globulins to such a degree that the albumin/globulin ratio is reversed. The increase in globulin is largely in the euglobulin fraction, although the pseudoglobulin increases somewhat.

4. The serum albumin returns to an essentially normal level in uncomplicated cases during the fifth week, but the serum globulin remains elevated in almost all patients for as long as three and a half months after onset of the disease.

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CHANGES IN CEREBROSPINAL FLUID PRESSURE UNDER THE INFLUENCE OF CONTINUOUS SUBARACHNOIDAL INFUSION OF NORMAL SALINE

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In 1944 we described a method (1) for the production of analgesia by the continuous subarachnoid infusion of dilute solution of local anesthetic agents (procaine 0.5%). The method involved the introduction into the subarachnoid space of larger volumes of fluids than those used in earlier methods. Manometric measurements of the spinal fluid pressure in the course of the first few anesthetics administered by the new method showed that at the rate of infusion employed (4 to 10 drops per minute) the spinal fluid pressure was elevated only moderately, and the elevation caused no symptoms (2). Nevertheless it seemed that it would be of interest to study the effect of subarachnoidal infusion on a group of non-anesthetized patients; to observe the changes of the spinal fluid pressure with different rates of infusion and find out what, if any, symptoms accompany a sustained pressure.

MATERIAL AND METHOD

This study was carried out in 15 male and five female patients from the Neurosurgical Service of the Massachusetts General Hospital. The ages ranged between 16 and 70 years. All patients needed lumbar puncture for diagnostic purposes. Patients whose findings indicated disturbed cerebrospinal fluid dynamics were excluded from the study.

The selected patients received 0.2 gm. of phenobarbital by mouth one and one-half hours before the start of the experiment. Lumbar puncture was accomplished and the spinal needle connected to the apparatus for continuous subarachnoid infusion in the manner described in previous communications (1, 2) (Figure 1).

The patient was then placed in the supine position and initial levels of spinal fluid pressure, pulse rate and blood pressure were taken and recorded. The spinal fluid pressures measured with this setup were somewhat higher than those usually obtained with the patient in the lateral horizontal position. This was due to the fact that the zero point of the manometer was adjusted to the level of the skin of the back, and that the head was somewhat elevated by the small pillow. Since we were more interested in changes of the spinal fluid pressure than in

absolute value this arrangement was selected, because it offered greater comfort to the patient and uniform conditions of observation. Resting level of the spinal fluid pressure was determined by repeated readings, and spinal fluid dynamics were tested by observing the effect of jugular compression on spinal fluid pressure. Normal saline and Hartman solution were used alternately for the subarachnoid infusion. No difference could be observed between the two fluids in their effect on spinal fluid pressure changes. The specific gravity of the spinal fluid and the infused saline was not considered in the pressure measurements.

Fluid was administered intrathecally at the rate of 4, 8, 12, 16 and 60 drops per minute to four persons in each

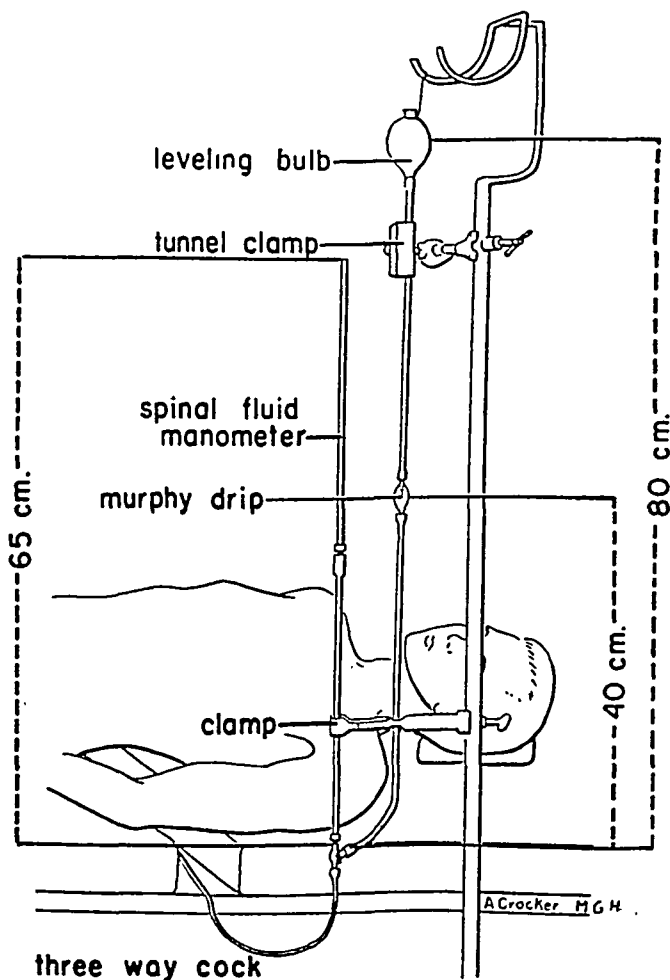


FIG. 1. DIAGRAM OF THE APPARATUS USED

TABLE I

The relationship between the rates of infusion and the approximate manometric pressure, measured under conditions similar to those in the subarachnoid space

Rate of drops per min.	Pressure in mm. H ₂ O
4	270
8	330
12	380
16	390
60	590

group. The dripper used delivered 20 drops to one cc. When the rate was between 4 and 16 drops per minute the subarachnoid infusion was continued for 60 minutes. At the end of this period the rate was increased for a varying length of time (10 to 20 minutes) to 30–80 drops per minute. When the starting rate was 60 drops per minute the infusion was continued for 20 minutes. Spinal fluid pressure, pulse rate, and blood pressure were recorded every 10 minutes and in some of the earlier experiments neurological signs and symptoms were also observed.¹ The patients were instructed to report any changes in their sensations. These were also recorded. After the infusion was discontinued, spinal fluid pressure was observed every minute until it became more or less stabilized. If this level was considerably higher than the initial spinal fluid pressure of the patient, spinal fluid was removed at the rate of 2 cc. per minute until normal or slightly higher than normal pressure was obtained. The spontaneous remission of the spinal fluid pressure of the four persons, in whom the initial rate of infusion was 60 drops per minute, was observed for 30 minutes. In these patients, spinal fluid pressure readings were made every minute in the first five minutes and every five minutes thereafter for 25 minutes.

Besides the quantity of the fluid administered it also seemed necessary to know the pressure under which the fluid entered the subarachnoid space. Because of the complexity of the physical factors involved, this pressure could not be calculated readily and had to be determined experimentally. An approximate measure of the pressures involved could be obtained by placing the Hingson needle at the end of the assembled apparatus in a glass tube closed at one end, containing normal saline solution. The level of the saline in the tube was regulated so that the hydrostatic pressure within it was the same as the average spinal fluid pressure at which a dynamic equilibrium developed between the quantity of the fluid administered and the quantity absorbed by the cerebrospinal system. Naturally this pressure varied with the different rates of infusion. Table I presents the manometric pressures of the system obtained at the different rates of infusion under the experimental conditions described.

RESULTS

The spinal fluid pressures of all the experimental subjects before the start of the infusion,

¹ We wish to express our gratitude to Dr. J. J. Michelsen who made the neurological observations in some of our experiments.

and every 10 minutes thereafter for 60 minutes, are presented in Table II. Figure 2 presents the combined spinal fluid pressure curves obtained at different rates of infusion. The first four curves show similar features. All four indicate a steady rise of pressure for 40 minutes, and all four curves become more or less parallel with the X axis, indicating that development of equilibrium occurs between the quantity of the fluid administered, and amount absorbed by the cerebrospinal system. The equilibrium was reached at progressively higher levels as the rate of administration was increased, but the increase of the equilibrium pressure did not increase in straight proportion with the rate of administration. Equilibrium was reached with the infusion rate of 4 drops per minute at 265 mm. of H₂O pressure, with 8 drops per minute of 330 mm. pressure, with 12 drops per

TABLE II

Spinal fluid pressures before and after the subarachnoid infusion of normal saline at various rates

Drops/min.	Patient			Pressure in mm. H ₂ O						
	Name	Sex	Age	Before infusion	10'	20'	30'	40'	50'	60'
4	J. D.		56	210	200	230	230	240	270	270
	M. M.		69	210	240	240	240	275	250	240
	A. S.		57	250	255	260	270	290	280	280
	J. S.		46	250	240	250	300	—	—	—
	Average			230	234	245	260	268	266	263
8	K. D.		70	190	185	340	340	380	380	370
	E. G.		51	230	250	270	300	330	330	330
	H. M.		57	240	265	260	280	300	330	320
	R. B.		16	250	280	250	280	270	290	300
	Average			230	245	280	300	320	330	330
12	A. H.		38	180	200	230	280	320	320	340
	A. M.		54	180	200	240	300	320	350	340
	F. T.		70	200	230	260	310	350	340	320
	F. C.		59	230	300	390	430	430	430	430
	Average			197	230	280	320	355	360	357
16	L. M.		69	180	200	220	250	300	—	—
	I. F.		48	250	340	360	370	370	380	380
	N. L.		58	260	350	370	400	380	370	370
	H. K.		52	250	260	320	380	410	440	430
	Average			235	288	318	350	365	397	393
60	F. S.		34	300	500	570				
	H. M.		26	220	420	550				
	W. H.		44	220	400	550				
	J. W.		54	220	450	640				
	Average			240	442	578				

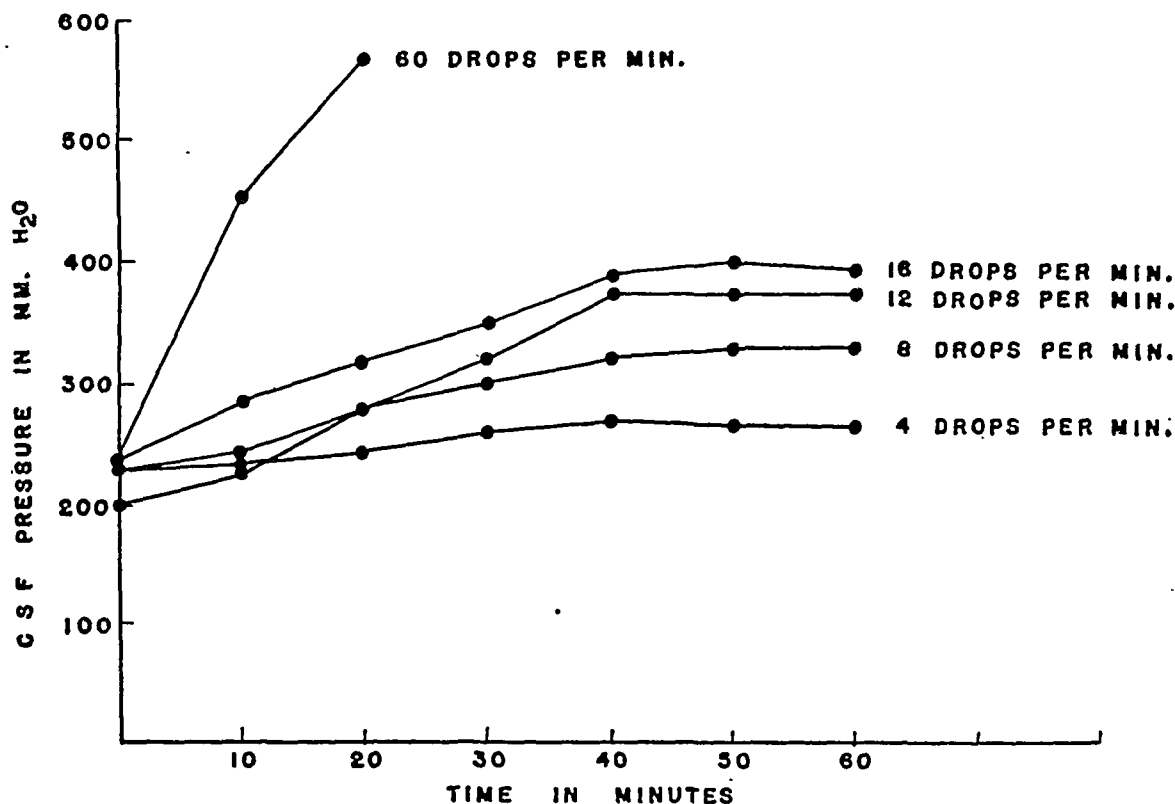


FIG. 2. SPINAL PRESSURE CURVES AT DIFFERENT RATES OF INFUSION
Each curve represents the mathematical average of four experiments.

minute at 360 mm. pressure, with 16 drops per minute at 395 mm. pressure.

When the subarachnoid infusion was administered at the rate of 60 drops per minute, the spinal fluid pressure rose rapidly and in 20 minutes it reached an average pressure of 570 mm. H₂O. The infusion was discontinued at this point because of the possible dangers of further increase in pressure. Immediately after the infusion was discontinued, the spinal fluid pressure was followed closely. These observations were recorded in Figure 3, which represents the combined spinal fluid curves of four patients during and after the subarachnoid infusion of normal saline at the rate of 60 drops per minute. It can be seen that the cessation of the infusion was followed by a sharp drop in the spinal fluid pressure. Within four minutes the pressure decreased from 580 mm. H₂O to 350 mm. of H₂O. From this time on, the pressure decrease was more gradual and 20 minutes after the end of the infusion the average pressure decreased to 295 mm. H₂O. In the last ten minutes of the observation period the spinal fluid pressure remained practically unchanged. At this point the removal of 4 to 10 cc. of spinal fluid at the

rate of 2 cc. per minute returned the pressure to normal.

Following the infusions at rates of 4, 8, 12, and 16 drops per minute, the pressure was similarly increased to high levels at the end of the one-hour observation period, by the rapid infusion of saline over periods of ten to 20 minutes. After infusion was stopped the spinal fluid pressure decreased rapidly during the first four to five minutes, then the decrease became more gradual. Removal of 4 to 24 cc. of spinal fluid, at the rate of 2 cc. per minute, started at four to ten minutes after the end of the infusion, returned the spinal fluid pressures to near normal values.

Neurological observations carried out on a few of the first cases revealed no significant findings. Neither were any remarkable changes observed in pulse rate, respiration or blood pressure. Mild symptoms were observed in nine out of the 20 cases, and more marked symptoms developed in one patient. The symptoms developed in most cases when, at the end of the one-hour observation period, the rate of infusion was markedly increased. The most frequent complaints were tired feeling, numbness, tingling and dull pain in the

legs, gluteal region or low back; in two cases similar symptoms were observed in the arms. Mild headache developed in two cases. In none of these nine cases were the symptoms severe enough to make necessary the premature termination of the infusion. All symptoms disappeared promptly when the infusion was discontinued or a few cubic centimeters of spinal fluid were removed. The only exception was patient A. S. who, after an uneventful observation period of one hour and a half, developed pain in the legs and arms when the rate of infusion was increased to 80 drops per minute. The patient suddenly became panicky, dyspneic, and the pulse rate jumped from 76 to 144. The spinal needle became dislodged and it was impossible to replace it because of the patient's restlessness. Eleven mgs. of morphine were administered intravenously and after the patient quieted down the needle was reintroduced and 30 cc. of fluid were removed. The spinal fluid pressure returned to normal level and symptoms were relieved.

No after-effects (headache, backache, etc.) occurred in any of the 20 patients.

COMMENTS

The mechanism of the spinal fluid dynamics cannot as yet be considered clarified in every respect. It might not be superfluous to consider briefly some of the prevalent views on this subject before attempting to interpret our experimental findings.

It is generally accepted, that most of the cerebrospinal fluid is produced by the choroid plexuses of the lateral ventricles (3). Opinions differ as to whether the production of the cerebrospinal fluid is a secretion (4), or a filtration process (5). The cerebrospinal fluid produced in the lateral ventricles flows through the ventricles and reaches the outer surface of the medulla oblongata through the foramina Luschka. The existence of the foramen Magendi is doubtful in man (6). From here, most of the cerebrospinal fluid flows forward into the cisterna basalis and then into the subarachnoid space overlying the hemispheres. Thence a smaller portion flows into the cisterna magna and on into the spinal subarachnoid space. The absorption of the cerebrospinal fluid occurs through the arachnoid villi (Pacchionian bodies) projecting into the venous sinuses of the dura.

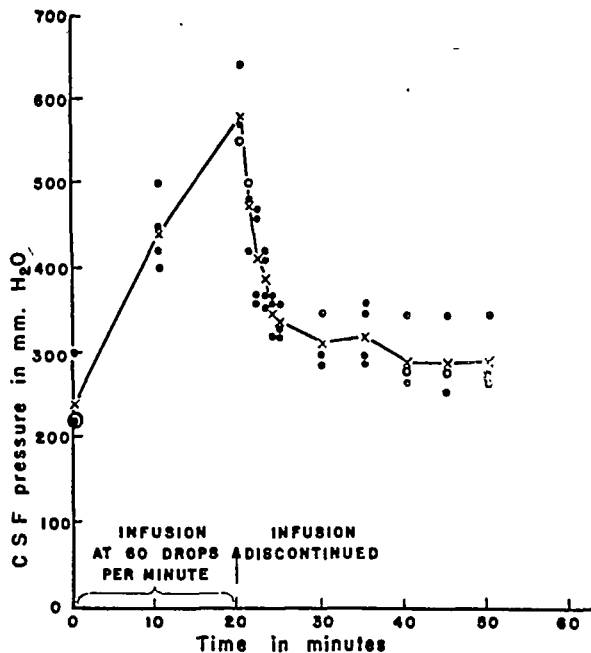


FIG. 3. COMBINED SPINAL FLUID CURVES OF FOUR PATIENTS DURING AND AFTER THE SUBARACHNOID INFUSION OF NORMAL SALINE, AT THE RATE OF 60 DROPS PER MINUTE

According to Weed (7) the absorption is due to the hydrostatic pressure of the cerebrospinal fluid (15–33 mm. H₂O) and to the osmotic pressure difference between the cerebrospinal fluid and the blood (250 to 300 mm. H₂O). Normal pressure is due to an equilibrium between production and absorption (O'Connell) (3). Dandy and Blackfan (8) stated that normally 500 to 800 cc. of cerebrospinal fluid is produced daily and according to Masserman (1934) (9) if a small quantity of spinal fluid is removed it is replaced at the rate of 0.3 cc. per minute. Weed (10) found that the higher the injection pressure the more rapid the rate of absorption of normal saline from the subarachnoid space. The rate of absorption in dogs showed a linear relationship to pressure in the lower pressure ranges. With higher pressures the rate increased out of proportion. No difference was found between the rate of absorption of distilled water, Locke, and double Locke solutions (Mortensen and Weed) (11).

The dural sac of man can be considered as a fairly, but not absolutely rigid tube (Weed) (12). Consequently if fluid is administered intrathecally dilatation of the subarachnoid space can only com-

compensate for the increased volume to a limited extent. Other possibilities of compensation are the increased rate of absorption through the arachnoid villi and displacement of blood (mostly venous) from the cerebrospinal circulation. Masserman and Schaller (13) found that in cadavers relatively sudden and large variations in cerebrospinal fluid pressure were fairly well transmitted from one part of the system to the other. There is no reason to believe that this should be different in the living.

The finding that at the infusion rate of 4 to 16 drops (0.2 cc. to 0.8 cc.) per minute the pressure only rose to a certain point, at which point an equilibrium developed between the amount of fluid infused and absorbed, is in agreement with the data of Weed (10). Our results also indicate that, as was pointed out by Weed (10), the relationship between cerebrospinal fluid pressure and absorption is not linear. The increase in the rate of absorption as measured by the rise in spinal fluid pressure was relatively large at first, then became smaller, then again larger and finally smaller again as the infusion rate increased from 4 drops per minute to 60 drops per minute. The changes that occur in the cerebrospinal fluid dynamics while the infusion rate is increased from 4 to 60 drops per minute could perhaps be imagined in the following way: when the rate of infusion was 4 to 16 drops per minute, the relatively small quantities infused into the subarachnoid space were easily disposed of—probably by an increased rate of absorption alone. When the infusion rate was increased to 60 drops per minute, the elevation of the spinal fluid pressure could not establish an equilibrium between infusion and resorption at clinically safe pressure levels. This indicates that in this case the cerebrospinal fluid dynamics had to resort to other factors to compensate for the increased amount of cerebrospinal fluid. Since the brain is practically incompressible, the only other two possibilities for compensation were the expansion of the dural sac and the expression of blood from the vessels (mostly dural sinuses) of the central system. The elasticity of the dura is very low, hence expression of blood had to be the dominant factor. The shape of the spinal fluid pressure curve after the cessation of the infusion at the rate of 60 drops per minute seems to corroborate this assumption. The

rapid fall in pressure in the first few minutes apparently corresponds to the absorption of that part of the cerebrospinal fluid that was present at the expense of the blood expressed from the vessels of the central nervous system and because of the possible distension of the dura. It might be assumed that from that point there are no gross changes in the volume of the vessels or in the distension of the dural sac, the decrease of the cerebrospinal fluid pressure progresses more slowly, and the pressure seems to be stabilized around 300 mm. H_2O .

Seiro (14) in a somewhat different experimental setup measured the amount of fluid that could be infused into the subarachnoid space in a given time (three or five minutes) at 400 mm. H_2O pressure. He also found that after stopping the infusion, the spinal fluid pressure dropped rapidly (150 mm. in 2 to 3 min.) at first and then more slowly.

Both Seiro (14) and Wolff and his associates (15) found very little or no clinical signs and symptoms that could be attributed to the elevation of the cerebrospinal fluid pressure. In one case where a cerebrospinal fluid pressure of 510 mm. of H_2O was maintained for ten minutes some discomfort was felt by the patient in the sacral region. In contrast to this, decrease of the spinal fluid pressure due to removal of fluid caused severe headache.

The method of study described in this paper might prove to be a useful tool in further studies of the dynamics of the cerebrospinal fluid. By changing the chemical composition of the fluid administered in the subarachnoid space it might be possible to find out whether or not the resorption of the cerebrospinal fluid is a filtration process. The selective absorption or retention of certain substances would indicate that other biological factors also have a part in resorption. It might also be possible to utilize the method as a neurological diagnostic procedure that would give quantitative information as to the changes of absorption of the cerebrospinal fluid in various pathological conditions.

SUMMARY

1. Normal saline was administered intrathecally at the rates of 4 to 60 drops (0.2 to 3 cc.) per minute.

2. When the rate of infusion was between 4 and 16 drops per minute, an equilibrium developed between the elevated cerebrospinal fluid pressure and the rate of absorption of the cerebrospinal fluid. The equilibrium was reached at progressively higher levels as the rate of infusion was increased. The relationship between rate of absorption and pressure is not linear.

3. No equilibrium was obtained within clinically safe pressure limits when the rate of infusion was 60 drops per minute.

4. After the infusion was discontinued the spinal fluid pressure decreased at first rapidly (180 mm. of H_2O in four minutes), then more slowly.

5. The elevation of the cerebrospinal fluid pressure to between 500 and 600 mm. of H_2O for ten to 20 minutes produced mild symptoms in about half of the patients. No untoward after effects were observed in any of the 20 patients.

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BLOOD AND EXTRACELLULAR FLUID STUDIES IN CHRONIC MALNUTRITION IN INFANCY^{1, 2}

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One problem facing the Italian Medical Nutrition Mission in 1945-46 was the great number of infants and children suffering from severe and chronic malnutrition. Through lack of sanitation, milk control and proper food, cases of severe inanition were encountered quite frequently in southern Italy.

The obvious dehydration of these emaciated infants on one hand and the poor results obtained with intravenous fluid therapy on the other hand suggested that there existed a severe disturbance of the distribution of body water rather than a simple deficiency in body fluids. Studies under the actual conditions of a war-torn and poverty-stricken country seemed therefore to be desirable.

METHODS AND MATERIAL

Clinical material

Twenty-three cases of severe marasmus in infancy and six cases of chronic malnutrition in older children were examined and 55 observations were made. Prior to being admitted to the Annunziata Hospital in Naples these infants had suffered generally from several attacks of gastro-intestinal disturbances, particularly diarrhea and vomiting, which presumably caused their initial loss of

weight. With the coming of the colder season these diarrheal attacks ceased but the children failed to gain weight. Most of the time they suffered from anorexia and occasionally from vomiting. Many were afflicted with thrush, scabies, impetigo or bed sores. None of the children showed signs of nutritional edema. Their dietary regime consisted of milk mixtures or whole milk with the addition of flour. Their fluid intake was comparatively low since anorexia was prevalent and additional fluid in the form of tea was offered only occasionally.

After many weeks of failure to gain weight or continued weight loss, the majority of the children developed all the clinical signs of marasmus and died in a condition of extreme inanition or of intercurrent infections.

In order to determine the degree of malnutrition the actual weight of the child was compared with the height and weight of standard American children (1). Since these standard average values would produce a comparison with a hypothetical standard individual another method was also used which would allow a greater individualization. Therefore the "calculated weight" was established for all infants examined. This calculated weight (2) is based on the formula:

$$\frac{\text{chest circumference}^2}{K} \times \text{height} \times \text{spec. gravity.}$$

The measurements of the chest circumference and the height are the only data required. Calculations are simplified by the use of a nomogram. Chest circumferences were measured at xiphoid level and at quiet expiration. Height was measured in prone or supine position. Body surface was determined from the same data by the use of the Du Bois formula (3).

Biochemical methods

For the determination of hemoglobin, hematocrit and plasma protein the specific gravity method was used (4). Albumin and globulin were determined with the method of Greenberg (5). Non-protein nitrogen determinations were carried out with the method of Wong-Buell and Archibald (6).

For the simultaneous determination of plasma volume and extracellular fluid space the method of Gregersen and Stewart (7) was adopted and modified for the Coleman Junior Clinical Spectrophotometer Model 6. For the children up to 6 kg., 1 mg. of the dye T-1824 was injected; for children from 6 to 20 kg., 3.3 mg.; and for older children above 20 kg., the usual dose for adults of 10 mg. was injected. To determine the extracellular fluid space a sterile solution of 10 per cent Na SCN was added to the dye in proportions not to exceed 20 mg. per

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kg. of weight. For children up to 6 kg., 60 mg. Na SCN were injected; from 6 to 20 kg., 200 mg.; and for children above 20 kg., 400 mg. All intravenous injections and withdrawals of blood were made in the longitudinal sinus. No accidents or reactions were encountered.

For the determinations of sodium in the plasma the method of Butler and Tuthill (8) was used. For chloride in the plasma the method of Van Slyke and Sendroy (9) was employed.

Material

Table I shows all data used for the analysis of the encountered condition.

Of the 23 infants examined 14 were males and nine were females. The age ranged from six weeks to two years and nine months. Only three infants were older than one year.

As noted above, all children suffered from chronic malnutrition. In 18 infants the cause of malnutrition

TABLE I

No.	Age			Diagnosis	Weight	Hemogl.	Plasma prot.	Plasma vol.	T-1824 dis. rate	Extracell. fl.
	yr.	mo.	wk.							
30		2	1	Malnutr.	2.9	8.6	7.7	207	31.5	909
1109		1	1	Malnutr.	2.7	11.8	5.1			1333
0522		4	3	Malnutr.	2.8	8.1	4.4	234	28.0	1090
Sc.		14		Malnutr.	4.5	11.4	6.1	621		1935
Sc.		14	2	Malnutr.	4.1	14.0	5.8	243	20.5	1579
Sc.		14	3	Malnutr.	4.2	11.2	5.8			1250
0627		3		Malnutr.	2.7	10.5	5.1	170	20.2	1032
1082		3	2	Malnutr.	2.4	9.3	6.8	150	6.0	699
1082		3	2	Malnutr.	2.4	8.9	6.1	169	16.0	863
336		20		Malnutr.	5.2	6.9	7.2	387	13.0	1818
336		24		Malnutr.	7.2	7.3	6.8			2927
1091		3	2	Malnutr.	3.5	11.7	6.8	174	7.5	883
1091		3	3	Malnutr.	3.6	9.7	5.5	222	28.0	1304
1091		4	3	Malnutr.	3.4	10.9	6.2	213	18.0	1223
0604		4	1	Malnutr.	2.9	10.9	7.2	189	11.5	909
0604		7	1	Malnutr.	4.2	12.0	5.8	312	9.0	1463
Ch.		9	2	Brain tumor	9.2	14.0	5.8	645	13.6	4190
0700		3	1	Malnutr.	2.5	10.7	5.8	165	19.5	882
Lang.		3		Malnutr.	2.9	9.7	5.8	330	14.0	993
Lang.		3	2	Malnutr.	3.3	10.2	5.5	234	12.0	1176
Lang.		4	2	Malnutr.	3.6	10.5	5.8	310	22.5	1220
Madd.		2	3	Pylor. sten.	3.7	11.2	6.1	216	19.0	1304
Madd.		3		Pylor. sten.	3.7	11.8	6.2	238	22.0	1250
1110		2	1	Malnutr.	2.7	9.4	5.8	226	4.2	1250
1110		2	3	Malnutr.	2.8	8.5	6.5	216	14.0	1177
Add.		1	2	Pylor. sten.	2.8	13.1	5.1	177		1071
Mir.		7	2	Malnutr.	4.0	11.6	4.8			1580
Mir.		8		Malnutr.	4.4	10.3	5.8	360		1764
Mir.		8	2	Malnutr.	4.9	8.8	5.8	351		2222
P18		1		Malnutr.	2.9	15.6	5.5			1200
P18		1	2	Malnutr.	3.3	11.6	4.8	372	3.6	1500
Cin.		2	3	Pylor. sten.	3.5	11.2	5.1	222	22.5	1540
0850		2	3	Malnutr.	2.8	11.6	4.8			1250
0850		3	1	Malnutr.	3.8	10.8	5.1			1154
343		12	4	Malnutr.	4.8	7.8	4.4	381	20.5	
343		12	4	Malnutr.	4.9	9.4	4.8	381	24.0	1818
343		12	5	Malnutr.	5.2	9.5	5.6	342	34.0	2166
343		12	8	Malnutr.	7.6	9.9	6.8	450		2857
398	9	2		Malnutr.	25.9	13.8	7.2	1480	9.6	8166
398	9	3		Malnutr.	26.4	13.4	7.2	1390	9.0	7407
399	9	4		Malnutr.	22.1	11.8	7.2	1870		7339
399	9	5		Malnutr.	24.5	12.2	6.8	1500	13.8	7547
402	6	8		Malnutr.	18.7	13.2	6.8	1180	3.7	5519
402	6	9		Malnutr.	19.0	14.2	7.2	907	15.0	5555
403	10	2		Malnutr.	27.5	13.3	6.8	1600	11.1	7700
403	10	3		Malnutr.	27.7	12.9	7.2	1580	4.8	7273
433	9	6		Malnutr.	24.5	13.8	7.2	1260	6.9	5940
433	9	7		Malnutr.	25.4	13.4	7.2	1100	4.0	6154
435	10	2		Malnutr.	27.0	12.6	6.8	1640	10.5	7690
435	10	3		Malnutr.	28.9	13.9	7.2	1460	4.5	7500
63	2	2		Malnutr.	8.8	12.2	6.8	515		
63	2	9		Malnutr.	9.8	11.8	6.8	660	9.0	3333
Div.		5	3	Wilms tumor	5.9	8.2	7.7	330	3.5	1765
Div.		5	3	Wilms tumor	5.9	7.5	7.2	345		
60	2	1		Malnutr.	9.3	11.7	6.8	660	8.0	3030

TABLE II
Physical measurements of 13 marasmic infants

No.	Age	Weight	Height	Chest circum.	Calc. weight	American Standard weight	Surface	Retardation in development	
	mo.	kg.	cm.	cm.	kg.	kg.	sq. m.	mo.	wk.
0522	4.3	2.8	53.5	32.9	3.6	6.8	0.200	3	3
Sc.	14.0	4.4	66.0	39.4	6.4	9.8	0.280	12	2
0627	3.0	2.7	50.5	33.6	3.6	5.7	0.190	2	3
1082	3.2	2.4	48.5	29.5	2.8	6.0	0.174	3	0
336	20.0	5.2	64.0	39.8	6.4	12.5	0.300	17	3
1091	3.2	3.5	54.5	33.0	3.7	6.4	0.223	2	3
0604	3.3	2.9	51.0	33.8	3.7	6.4	0.195	3	2
0700	3.1	2.5	46.5	28.9	2.5	5.8	0.172	3	1
Lang.	3.0	2.9	51.5	34.8	3.9	6.1	0.196	2	3
1110	2.1	2.8	49.0	33.3	3.5	5.5	0.186	2	0
Mir.	7.2	4.0	57.5	37.8	5.2	8.6	0.246	6	3
0850	2.3	2.9	50.5	32.4	3.8	5.5	0.193	2	1
343	13.0	4.8	60.5	39.2	5.8	10.8	0.268	11	1
Average	6.0	3.4	54.1	34.5	4.2	7.3	0.217	5	2
Normal	6.2	7.3	66.0	43.5	7.6	7.3	0.350		
% Difference		-53.9	-18.1	-20.7	-44.8		-38	-91	

could be traced to previous intestinal disturbances; three of the children suffered from pyloric stenosis, one child from a brain tumor and one child from a Wilms tumor.

By eliminating the older children and the children in whom malnutrition was caused by other pathology than preceding diarrheal disease, a group of 13 children can be extracted whose age and clinical pathology are comparable and who lend themselves to a more detailed analysis.

RESULTS

Physical status

Table II shows the changes in body weight, height and surface in these 13 selected children who had undergone severe malnutrition.

The physical measurements show that during chronic malnutrition these infants had lost, or failed to achieve on the average, 53.9 per cent of their weight, 44.8 per cent of their calculated weight, 18.1 per cent of the height, 20.7 per cent of the chest circumference and 38 per cent of the body surface if compared with the standard values of the same age. The Grid method (10) for the evaluation of speed and direction of development of each individual child could not be employed to its full advantage because the time of observation was not long enough. If it is permissible, however, to determine the developmental level line

TABLE III
Hematocrit and hemoglobin of 13 marasmic infants

No.	Hematocrit	Hemoglobin	Total circul. red cell mass	Total circul. hemogl.	Total circul. hemogl.	Total circul. hemogl.	Total circul. hemogl.	Total circul. hemogl.
	%	gm. %	cc.	gm.	per kg. act. wt.	per kg. calc. wt.	per cm. ht.	per sq.m. surface
0522	24.0	8.1	73.7	24.9	8.9	6.9	0.46	124.5
Sc.	33.4	11.4	311.3	107.2	24.4	16.7	1.62	375.6
0627	31.0	10.5	76.3	25.8	9.6	7.2	0.51	135.7
1082	27.3	9.3	55.1	18.8	7.8	6.7	0.38	110.5
336	20.5	6.9	99.6	33.5	6.4	5.3	0.52	111.6
1019	34.4	11.7	90.5	30.8	8.8	8.3	0.56	138.1
0604	32.2	10.7	89.2	29.6	10.0	8.0	0.58	151.7
0700	31.5	10.7	75.6	25.7	10.0	10.0	0.55	149.4
Lang.	28.5	9.7	131.1	44.6	15.4	11.0	0.86	227.5
1110	27.5	9.4	86.1	29.4	10.0	8.4	0.60	158.0
Mir.	34.0	11.6	176.8	60.3	15.1	11.8	1.00	245.1
0850	34.0	11.6						
343	24.0	7.8	120.0	39.0	8.1	6.8	0.64	145.6
Average	29.4	9.9	116.1	39.1	11.2	8.9	0.69	172.7
Normal	35.5	11.8	163.3	54.3	7.4	7.2	0.81	155.1
% Difference	-17.2	-16.1	-28.8	-27.9	+51.3	+23.6	-14.7	+11.3

for each child from the age, weight and height figures and to compare their average with the performance standard on the speed of development for babies of "average" advancement of the same age, then the projection from the auxodrome on the age scale results in a retardation of development of five and a half months.

Hematocrit and hemoglobin

The results of hematocrit and hemoglobin determination (Table III) show that there is a decrease of 17.2 per cent in the hematocrit and of 16.1 per cent in the hemoglobin concentration as compared with normal values of hematocrit (11) and hemoglobin (12) for this age. This decrease in hemoglobin production became more marked when the value of the total circulating hemoglobin was derived from hemoglobin concentration and blood volume. Also the total circulating red cell mass was decreased. The ratio of total circulating hemoglobin to units of weight, height and body surface shows that in infants hemoglobin does not seem to be destroyed to a greater degree than other body tissue.

Plasma proteins

Plasma protein concentration (Table IV) shows a decrease of 6.4 per cent if compared with normal values for this age (13). If the total circulating

TABLE IV
Plasma protein in 13 marasmic infants

No.	Plasma protein	Total circul. plasma protein	Total circul. plasma protein	Total circul. plasma protein	Total circul. plasma protein	Total circul. plasma protein
	gm. %	gm.	per kg. act. wt.	per kg. calc. wt.	per cm. ht.	per sq.m. surface
0522	4.47	10.5	3.7	2.9	0.19	52.5
Sc.	6.18	39.0	8.8	6.1	0.59	13.9
0627	5.12	8.7	3.2	2.4	0.17	45.8
1082	6.83	10.2	4.2	3.6	0.21	58.6
336	7.18	27.8	5.3	4.3	0.43	92.3
1091	6.83	11.9	3.4	3.2	0.22	53.4
0604	7.18	13.6	4.7	3.6	0.26	69.7
0700	5.83	10.0	4.0	4.0	0.22	58.1
Lang.	5.83	19.2	6.5	4.8	0.37	97.9
1110	5.81	13.1	4.7	3.7	0.26	70.5
Mir.	4.80	17.3	4.2	3.3	0.30	70.3
0850	4.80					
343	4.43	16.9	3.5	2.9	0.28	63.1
Average	5.8	16.5	4.7	3.7	0.29	62.2
Normal	6.2	17.4	2.3	2.3	0.26	49.7
% Difference	-6.4	-5.1	+104.3	+60.8	+11.6	+25.1

TABLE V

Plasma albumin and globulin in eight marasmic infants

No.	Albumin	Globulin	A/G
	gm. %	gm. %	
0522	3.01	2.04	1.47
1082	4.52	2.10	2.15
336	4.43	2.53	1.75
0604	3.49	2.48	1.41
Lang.	3.62	2.14	1.69
1110	3.99	2.31	1.71
Mir.	3.00	2.12	1.40
343	3.09	1.67	1.80
Average	3.64	2.17	1.64
Normal	4.6	1.30	3.50
% Difference	-20.8	+66.9	-53.1

plasma protein is calculated by multiplication with the plasma volume then the decrease amounts to 5.1 per cent. This is due to an increased plasma volume as can be shown also by the relation of total circulating plasma protein to the unit of body weight, height and surface.

The determination of the albumin and globulin fractions (Table V) shows that the decrease in the plasma protein concentration is due entirely to the decrease in albumin concentration. The globulin fraction shows an increase which is equal to the reduction in the albumin fraction. Thus the albumin-globulin ratio is reduced to half of its normal value for this age group (13).

The non-protein nitrogen was in normal ranges in all except two cases of extreme inanition in which it rose to levels of 41 and 49 mg. per cent.

Plasma and blood volume

The determination of plasma volumes (Table VI) shows that the absolute value did not change significantly during severe malnutrition as compared with normal values of the same age group (14). If the absolute value is related to the actual weight and to body surface, however, the plasma volume exceeds by far the normal values.

The absolute values for the blood volume (Table VII) are 14.1 per cent lower in severe malnutrition in infancy if compared with normal values of the same age group (14). If the blood volume is related to the unit of body weight or body surface an increase of 77.7 per cent and 32.8 per cent results. No significant change occurs in the relation of blood volume to unit of body height.

TABLE VI
Plasma volume in 13 marasmic infants

No.	Plasma volume	Plasma volume	Plasma volume	Plasma volume	Plasma volume	Plasma volume
	cc.	per kg. act. wt.	per kg. calc. wt.	per kg. stand. wt.	per cm. ht.	per sq.m. surface
0522	234	83	67	34	4.3	1170.0
Sc.	621	142	95	63	9.4	2928.6
0627	170	62	47	24	3.3	894.7
1082	150	62	52	25	3.1	862.0
336	387	74	60	36	6.0	1290.0
1091	174	47	46	27	3.2	780.2
0604	189	65	50	29	3.7	969.2
0700	165	66	66	28	3.7	959.3
Lang.	330	111	84	54	6.3	1683.6
1110	226	81	64	41	4.6	1215.0
Mir.	360	81	65	40	6.2	1463.4
0850						
343	381	79	64	35	6.3	1421.6
Average	282	79	63	36	5.0	1303.1
Normal	277.4	38	36	38	4.2	792.5
% Difference	+1.6	+105.2	+77.7		+19.0	+64.4

Extracellular fluid

Table VIII shows the results of the determinations of extracellular fluid space. There is a decrease of 57.5 per cent in the absolute values of the total extracellular fluid space if compared with the available fluid space of newborn infants (15). If related to the unit of weight no significant change occurs if the extracellular fluid space is compared with the same ratio of newborn infants. The extracellular fluid space in malnourished infants decreases to 49 per cent and to

TABLE VII
Blood volume in 13 marasmic infants

No.	Blood volume	Blood volume	Blood volume	Blood volume	Blood volume	Blood volume
	cc.	per kg. act. wt.	per kg. calc. wt.	per kg. stand. wt.	per cm. ht.	per sq.m. surface
0522	307	109	86	45	5.7	1535.0
Sc.	932	214	145	95	14.1	3328.5
0627	246	91	60	43	4.9	1294.7
1082	202	84	70	33	4.2	1160.9
336	486	93	76	45	7.5	1620.0
1091	263	75	69	41	4.8	1179.3
0604	277	95	73	42	5.4	1420.5
0700	240	96	96	41	5.2	1395.3
Lang.	460	155	117	75	8.9	2350.0
1110	313	113	89	56	6.4	1682.7
Mir.	520	117	94	57	9.0	2113.9
0850						
343	500	100	87	46	8.3	1865.7
Average	395	112	88	51	7.0	1745.5
Normal	460	63	61	63	6.9	1314.2
% Difference	-14.1	+77.7	+44.2	-19.0	+1.4	+32.8

TABLE VIII
Extracellular fluid in 13 marasmic infants

No.	EFS	EFS	EFS	EFS	EFS	EFS
	cc.	per kg. act. wt.	per kg. calc. wt.	per kg. stand. wt.	per cm. ht.	per sq.m. surface
0522	1090	38.9	31.5	16.0	20.3	5450.0
Sc.	1935	44.2	30.2	19.0	29.3	6910.5
0627	1032	38.2	28.6	18.1	20.4	5431.5
1082	699	29.1	24.6	11.6	14.4	4017.2
336	1818	35.0	29.0	17.0	28.4	6060.0
1091	883	25.9	23.5	13.9	16.2	3959.6
0604	909	31.3	24.2	14.2	17.8	4661.5
0700	882	35.2	35.2	15.0	18.9	5127.9
Lang.	993	33.6	25.4	16.2	19.2	5066.3
1110	1250	45.0	35.7	22.7	25.5	6720.4
Mir.	1580	39.7	32.0	19.6	27.4	6422.8
0850	1250	43.7	32.8	22.3	24.7	6476.6
343	1818	36.5	27.4	16.8	30.0	6783.6
Average	1241	36.6	29.2	17.1	22.5	5622.1
Normal	2920	40.0	38.4	40.0	44.2	8342.7
% Difference	57.5	-8.5	-23.9	-57.2	-49.0	-32.6

32.6 per cent if related to the unit of body height and surface.

If the absolute values for total circulating blood proteins and body fluid volumes are related to the various physical measurements, it is of interest to note that of all relations the one to calculated weight follows most closely the one to body surface.

Sodium and chloride

Sodium determinations in the plasma of six children showed slightly decreased values with an average of 135 m. Eq. per 1000 ml. Also the chloride in the plasma of 12 children was slightly reduced with an average value of 92.3 m. Eq. per 1000 ml.

CORRELATION OF FINDINGS

By establishing correlations between hemoglobin and plasma protein on one side and plasma volume and extracellular space on the other side, it is possible to elucidate the dynamics of these factors during different stages of malnutrition. Data taken from the study of all 29 malnourished children were used for these correlations.

Plasma protein—extracellular fluid

Figure 1 shows an inverse correlation between the level of plasma protein and the size of the extracellular space. The existence of this correlation, however, does not necessarily reflect on the

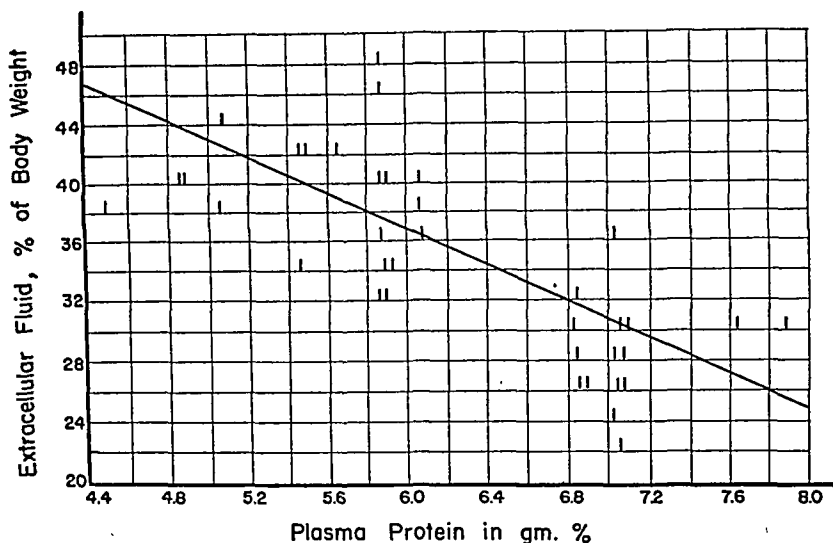


FIG. 1. CORRELATION BETWEEN PLASMA PROTEIN CONCENTRATION AND EXTRACELLULAR FLUID SPACE

etiology of nutritional edema. In the group of malnourished infants we studied clinical signs of edema were absent even when the extracellular fluid space exceeded 40 per cent of body weight.

Plasma protein—plasma volume

The inverse correlation between the level of plasma protein and the plasma volume in severe malnutrition shown in Figure 2 indicates that these two factors move in opposite directions. There-

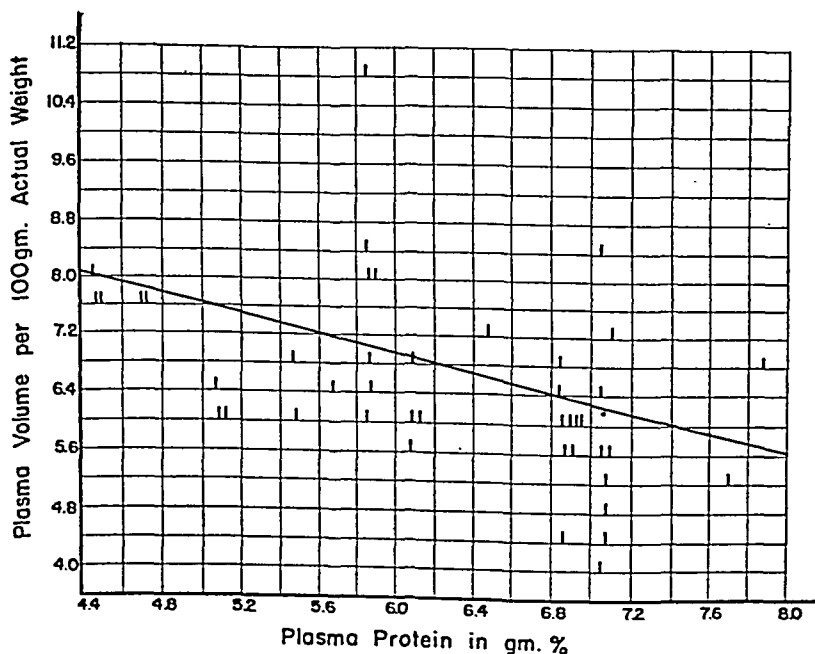


FIG. 2. CORRELATION BETWEEN PLASMA PROTEIN CONCENTRATION AND PLASMA VOLUME

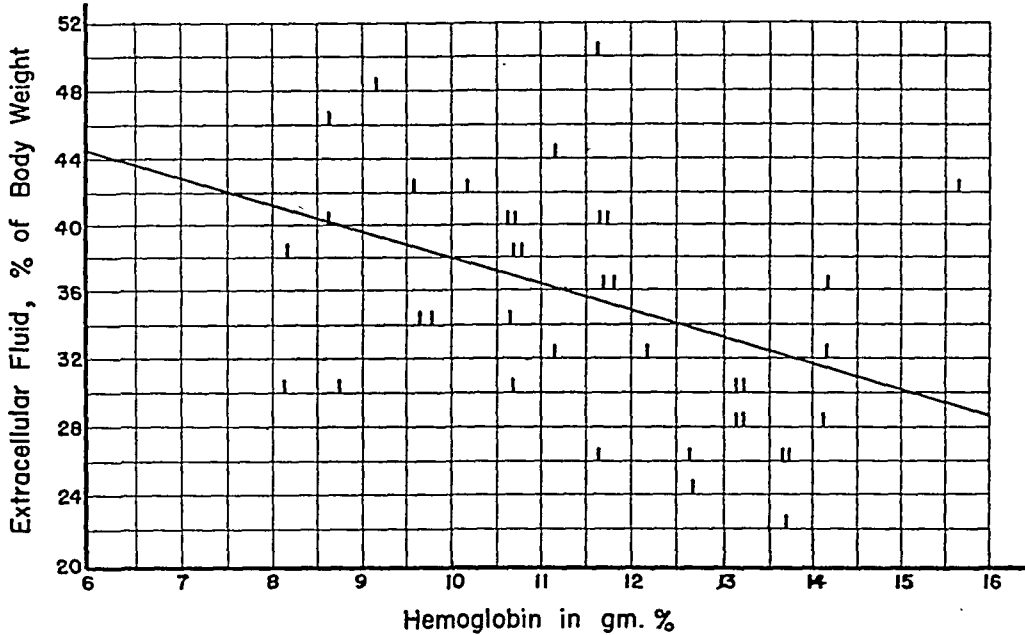


FIG. 3. CORRELATION BETWEEN HEMOGLOBIN CONCENTRATION AND EXTRACELLULAR FLUID SPACE

fore a low plasma protein level in malnutrition is related to a dilution factor as well as to reduced production.

Hemoglobin—extracellular fluid

The same considerations as expressed for the correlation on Figure 1 hold true also for the correlation between the concentration of hemoglobin

and the size of the extracellular space on Figure 3. This correlation also indicates more the increasing divergence of hemoglobin and extracellular space with the progressive severity of malnutrition than actual evidence for the etiology of the expansion of extracellular fluid space.

Plasma protein—disappearance rate of T-1824

In calculating the disappearance rate of the dye

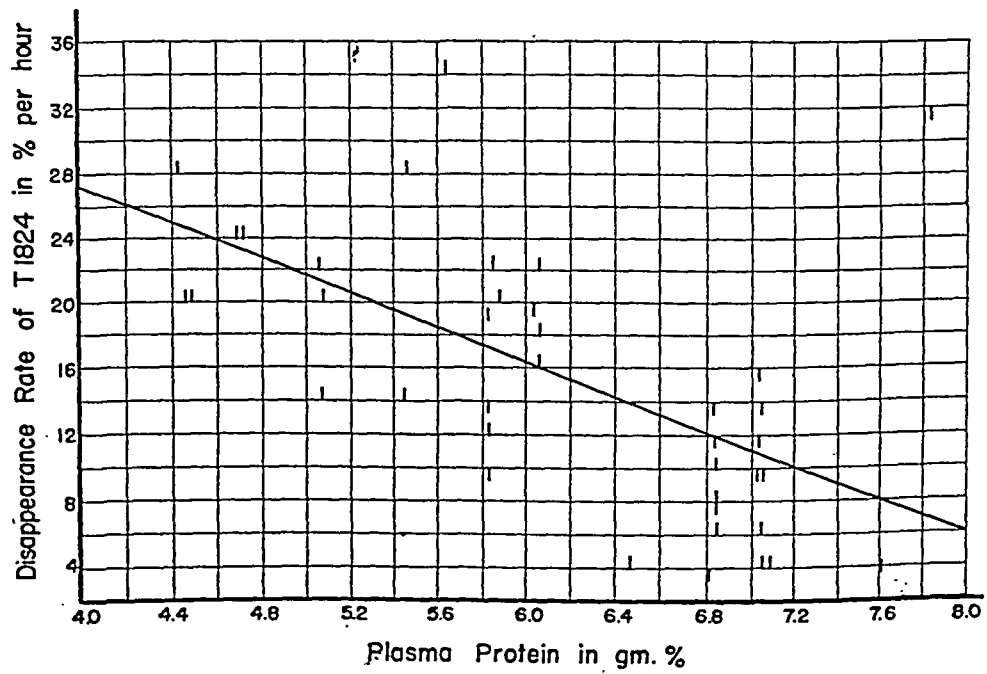


FIG. 4. CORRELATION BETWEEN PLASMA PROTEIN CONCENTRATION AND THE DISAPPEARANCE RATE OF THE DYE T-1824

T-1824 in the 20, 40 and 60 min. samples of plasma after the injection of the dye we have found a wide variation ranging up to 30 per cent in contrast to about 5.2 per cent per hour in normal adults (16). No information on the disappearance rate of the dye in normal infants at different ages is yet available. This change in the disappearance of the dye in severe malnutrition which averages 16.5 per cent, shows a definite inverse correlation to the level of plasma protein, as shown in Figure 4.

The reliability factors (statistically valuable if higher than 2) for the observed correlations are expressed in the following figures:

Plasma protein versus plasma volume.....	2.29
Plasma protein versus extracellular space.....	4.62
Hemoglobin versus extracellular space.....	2.55
Plasma protein versus disappearance rate.....	3.76

DISCUSSION

During the war and its aftermath new interest has been focused on the physiology and treatment of severe malnutrition and starvation. Although marasmus in infancy is prevalent in many parts of the world and has been known to pediatricians for a long time, little information has been forthcoming using newer methods of investigation.

Without doubt we are dealing with a very complex condition since a severe wastage of body tissue is superimposed upon blood constituents and body fluids still undergoing physiological changes. In addition, marasmus is not a simple deficiency disease because in its later stages an insufficient intake of food is not the main cause for progressive loss of body weight. Almost 30 years ago Marriott (17) observed that in many instances the food given to an infant is sufficient in calories but is either incompletely digested and absorbed or else is not completely utilized by the body after absorption.

Plasma proteins

Although there is a marked decrease in the concentration of hemoglobin and plasma protein in infantile marasmus the extremely low levels of blood proteins found in malnutrition and starvation in the adult are very rare (18). In contrast to the starving adult (19) where the loss of hemoglobin is relatively much greater than the loss

of other body tissue, the infant maintains its normal ratio of total circulating hemoglobin per unit of body weight and body surface. Only the ratio of total circulating hemoglobin to unit of body weight is reduced, a change which is also most marked in the adult.

Just as in the starving adult, the relative decrease in the total circulating plasma protein is less marked than that of the total circulating hemoglobin (19). However, because of the excessively increased plasma volume the ratio of total circulating plasma protein per unit of body weight, height and surface becomes larger than in the normal child of the same age. Whereas in the starving adult plasma protein is not greatly reduced relative to other body tissue (19), in the severely malnourished infant the rapid growth impulse combined with the increased plasma volume results in an increased ratio of plasma protein to body measurements.

The reduction in total circulating plasma protein is entirely due to a marked decrease in the albumin fraction. This observation has been made in all studies on malnutrition. Because of the slight increase in the globulin fraction, the total circulating globulin per unit of body measurement is above normal.

The normal values for blood non-protein nitrogen confirm previous observations in infants, as well as renal function tests on cases of starvation at the German concentration camp Belsen (20). Except in cases of gross edema, renal function has been found to be normal.

Blood volume

Blood volume studies in infants are not made routinely because of the technical difficulties involved. There exists therefore a lack of extensive physiological studies on blood volume in all age groups using methods which are comparable. Table IX shows a tabulation of blood and plasma volume values for normal infants of the same age as the children investigated in the present study.

This survey of the literature shows that the Evans Blue and carbon-monoxide methods give comparable results, since in normal infants of about six months of age the values for blood and plasma volume are in better agreement than the ones obtained by the vital red method. Similar

TABLE IX

Blood and plasma volume determinations of infants of six months of age by various authors

Author	Method	No. of cases	Blood volume	Plasma volume
			% of wt.	% of wt.
Lucas & Dearing (21)	Vital red	5	11.3	7.8
Bakwin & Rivkin (22)	Vital red	18	9.5	6.1
Darrow, Soule & Buckman (23)	Vital red	5	8.9	6.0
McIntosh (24)	CO	6	6.8	4.1
Brines, Gibson & Kunkel (14)	T 1824	3	5.8	3.6

results with the Evans Blue and carbon-monoxide methods have also been obtained in the same normal subjects (25, 26).

The interest in blood volume studies in malnourished infants is of long standing. Marriott and Perkins (27), using the vital red method, found a slightly reduced blood volume in malnourished children. Higher values, however, were obtained in infants who might have been considered as convalescing from marasmus. Bakwin and Rivkin (22) studied 30 infants of the age group comparable to our group and found a blood volume of 10 per cent and a plasma volume of 6.8 per cent which is slightly higher than the values for their normal children of the same age. Recently, controlled studies on human volunteers have confirmed these early observations on infants. Henschel, Taylor, Keys and Sturgeon (28) found after 24 weeks of semi-starvation a blood volume increase to 120 per cent of cc. per kg. of body weight—whereas the plasma volume showed an increase to 142.2 per cent. The same facts are born out by observations made on cases of severe and prolonged starvation at Belsen by Mollison (20). Here in subjects who had lost as much as 50 per cent of their body weight, in six male patients the average blood volume was 10.1 per cent and the plasma volume 6.8 per cent of the actual weight. In ten female subjects the blood volume was reduced to 7.9 per cent, but the plasma volume was increased to 6.8 per cent of actual body weight. Extensive studies have also been made on malnutrition in Indian prisoners of war in the Far East by Walters, Rossiter and Lehmann (19) of the Marasmus Research Team, India Command. Also here the relative decrease in plasma volume was not as great as that of body weight, and during

recovery the plasma volume per kg. of body weight rose to figures far in excess of normal. The total blood volume was slightly lower when the patients were admitted and during recovery it was never greatly in excess of normal.

Experimental studies by Utheim (18) on completely starved rabbits which were also deprived of water have shown that the blood volumes fell below the normal values when referred to body surface, as a result of water loss from the body. Correlated to the actual body weight, however, the blood volume remained fairly constant whereas the plasma volume increased slightly. By giving only enough food and water to prevent further loss of weight the plasma volume reached values above the normal for body weight as well as body surface. The same observations were made in hibernating woodchucks by Rasmussen and Rasmussen (29). The blood volume was lowest when the animals contained a maximum of fat. After dormancy and before food was available the percentage of blood volume was high. The most emaciated animals showed the highest percentage of blood volume in proportion of body weight.

Thus, all observations on blood and plasma volume in malnutrition of children, prisoners of war, human volunteers and of experimental animals agree that under the damaging influence of malnutrition and starvation the cellular elements of the body account for the severe loss of weight, whereas the plasma volume does not contract to the same extent and therefore shows a relative increase per unit of weight.

Extracellular fluid

The total extracellular fluid space undergoes physiological changes during infancy since the ratio of extra- to intracellular fluid diminishes from the fetal period to adulthood. Recent estimations of extracellular water with radio-active sodium showed values of 43.5 per cent in newborn children (15) as compared with 25 per cent of body weight of the adult determined also with tagged sodium (30). Values for normal infants of different ages are not yet available.

In malnutrition the hydration of the extracellular fluid can be expected to be normal or slightly reduced if water deprivation is also present (31), severely reduced if diarrhea is associated with it

(32) and greatly enlarged, manifesting itself in nutritional edema, if ample fluid and salt intake are permitted (33). The large increase in body water in malnourished infants has been observed 60 years ago by Ohlmüller (34) and has been measured with the thiocyanate method by Robinow and Hamilton (35). The well-controlled studies on human volunteers by Keys and his co-workers (33) have shown that after 12 weeks of semi-starvation the extracellular space as measured by the thiocyanate method may show an increase of over 40 per cent.

Edema

The infants studied in the present observations, whose fluid intake was low, did not show signs of edema. If intravenous fluid therapy had to be administered, however, the children developed edema very rapidly, proving that the inclination to edema in malnutrition can be made manifest if the means for edema are supplied.

In this connection an increase in capillary permeability which is suggested by the increase in the disappearance rate of the dye T-1824, assumes greater possible importance. Concerning the application of the blood volume method with Evans Blue this finding stresses the necessity of measurement of the disappearance rate of the dye whenever nutritional disturbances are present, and changes in the plasma albumin level are expected (36).

Recently serious doubt has been cast on the role which hypoproteinemia may play in the production of edema (37 to 39). Our observations in marasmic infants tend to strengthen the belief that hypoproteinemia and edema in malnutrition (40 to 42) are two co-existent but not causative factors in malnutrition. The low rate of blood flow which in some instances has been found from 80 to 90 per cent less than normal (18) and the reduced oxygen saturation and content of the venous blood (43), may prove to have a closer causative relation of nutritional edema than the low plasma protein concentration.

The treatment of the late stages of infantile marasmus is still unsatisfactory. The administration of intravenous fluid therapy in cases where food cannot be taken orally is contraindicated because plasma volume and extracellular fluid spaces tend to be high and more fluid and salt will lead

only to the formation of edema. Blood transfusions are of temporary value only except in cases of severe edema (19, 44). The observations reported here conform with the statement of Marriott (17)—that transfusions have a temporary value only unless the underlying cause of body destruction is found and remedied.

Liver biopsies in some of our cases failed to show any destructive lesions of the hepatic parenchyma.

Failure of absorption

Our experience with the oral administration of casein hydrolysates confirms rather the point of view that failure of absorption seems to be the essential lesion in the irreversible stages of starvation (45). In the case of a completely emaciated 13-year-old girl, well-being and gain in weight could be achieved as long as blood transfusions and large amounts of casein hydrolysates and dextrose by mouth were administered. When casein hydrolysates were no longer available we had to change to milk mixtures which were not tolerated and caused irritation, diarrhea and death. The construction of a diet which would not only meet all the increased nutritional requirements in malnourished children but would also attempt to substitute the specific intracellular substances lost, as successfully employed with potassium chloride infusions in infantile diarrhea (46), may eventually lead to absorption and retention of these elements and to an etiological treatment of infantile marasmus.

SUMMARY

Blood and extracellular fluid studies were carried out in 13 severely malnourished infants with the following results:

1. Compared with normal infants of the same age there occurred a loss of 53.9 per cent of actual weight, 44.8 per cent of calculated weight, 18.1 per cent of height and 38 per cent of body surface. In speed and direction of development, the marasmic infants of an average age of six months showed a retardation of five and a half months as measured by the Wetzell-Grid method.

2. The hematocrit and the hemoglobin concentration showed a decrease. The total circulat-

ing hemoglobin was reduced. The ratios of total circulating hemoglobin to unit of body weight, height and surface indicate that in malnourished infants hemoglobin is not destroyed to a greater degree than other body tissue.

3. The concentration of plasma protein and the total circulating plasma protein were reduced. The relation of total circulating plasma protein to unit of body weight, height and surface showed a marked increase.

4. The concentration of albumin was reduced, whereas the concentration of globulin showed an increase, resulting in a low albumin-globulin ratio.

5. The non-protein nitrogen was within normal ranges.

6. The absolute values of plasma volume did not change significantly if compared with normal values of the same age group. When this value was related to the actual weight and body surface, the plasma volume was far in excess of normal and its increase was less if related to body length.

7. The absolute values of blood volume were reduced. The ratio of blood volume to unit body weight and surface showed an increase. No significant change occurred in the relation of blood volume to unit of body height.

8. The disappearance rate of the dye T-1824 from the blood stream was increased.

9. The absolute values of extracellular fluid space were reduced. The relation of extracellular fluid space to unit of body weight did not change significantly. The extracellular fluid space decreased if related to unit of body height and surface.

10. The ratios of total circulating blood proteins and body fluid volumes to calculated weight—a value derived from chest circumference and body height only—followed closely the respective ratios to body surface.

11. The concentrations of sodium and chloride in the plasma were slightly reduced.

12. There existed an inverse correlation between plasma protein and extracellular fluid space, between plasma protein and plasma volume, between hemoglobin and extracellular fluid space and between plasma protein concentration and the disappearance rate of the dye T-1824.

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STUDIES ON GANGRENE FOLLOWING COLD INJURY. IX. THE EFFECT OF RUTIN AND OTHER CHEMICAL AGENTS ON THE COURSE OF EXPERIMENTAL FROSTBITE IN RABBITS ^{1,2}

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Methods for the production of controlled cold injury and the course of events following untreated injury of this type in rabbits have been described earlier in this series of papers (1, 2). Such injury leads rapidly to massive edema of the frost-bitten foot, drying and subsequent separation at the line to which the part was immersed in a freezing mixture. An invariable early feature of this controlled cold injury is loss into the injured region of large amounts of protein-containing fluid (3). The reversibility of this type of injury has been demonstrated by experiments in which the extent of tissue loss was greatly reduced or prevented by physical measures: rapid warming in water at +42° C. (4), rigid plaster or plastic casts or elastic pressure dressings (5). This reversibility emphasizes the importance of events subsequent to thawing as causal factors in the production of gangrene and suggests that the possible beneficial effects of chemical agents should be considered.

Treatment of frostbite by means of drugs has been generally unsuccessful (6 to 8). Recently Lange and Boyd (9) reported that tissue loss following experimental frostbite was reduced by treatment with heparin intravenously, but Quintanilla, Krusen and Essex (10) were unable to demonstrate the effectiveness of this anticoagulant. This paper reports some attempts to influence the course of controlled experimental cold injury in rabbits by means of a number of chemical agents. The flavonol glycoside, rutin, was found

to reduce the extent of tissue loss following frostbite of rabbit feet; the major part of this report is therefore devoted to this substance.

METHODS

Albino rabbits maintained on a diet of Albers' Family Ration, a dry commercial food in pellet form, were used in all experiments. The animals were anesthetized by the administration intraperitoneally of dial, 90 mgm. per kgm., supplemented with ether immediately before frostbite. The hair was removed from the ear or from one hind leg by close clipping. The distal 4 to 5 cm. of the ear, or the distal portion of the foot to the level of the tuberosity at the base of the fifth metatarsal, was frozen by immersion in a mixture of alcohol-water-ethylene glycol, cooled with solid CO₂ to -55° C. The ears were immersed for 60 to 90 seconds, the feet for three minutes. Complete details of this procedure have been given previously (1).

EXPERIMENTAL RESULTS

1. *Rutin*.³ Substances with Vitamin P activity have been reported to influence capillary permeability and fragility (11, 12). One of these substances, rutin, has become available recently in pure form. It has been reported to be effective in the treatment of capillary bleeding associated with hypertension in man (13, 14), and a recent report (15) indicates that it decreases the incidence of widespread petechiae and ecchymoses in dogs following X-irradiation. Because capillary injury is such a prominent feature in the lesions produced by exposure to severe cold, the effect of rutin was studied on standardized frostbite in rabbits in order to determine the possible prophylactic and/or therapeutic usefulness of this agent. In the following experiments rutin was adminis-

¹ A part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University. The study was also aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of the Stanford University School of Medicine.

² An abstract has appeared in Federation Proceedings, 1948, 7, 38.

³ The rutin used in these experiments was supplied by the Western Regional Research Laboratory, United States Department of Agriculture, through the courtesy of Dr. Floyd DeEds.

TABLE I

Frostbite of rabbit feet

Closely clipped foot immersed to level of tuberosity at the base of fifth metatarsal in freezing mixture at -54°C . for three minutes. Foot thawed in air.

Rutin administered by stomach tube daily. Dose 100 mgm. per kgm. in rabbits No. 243, 244, 245 and 246. Dose 50 mgm. per kgm. in others.

Animal number	Doses of rutin		Appearance of gangrene, days after frostbite		Result
	Before injury	After injury	Wet	Dry	
222	5	17	4	7	Lost toes; dorsum scarred
224	10	6	3	6	Lost toes; dorsum scarred
225	6	6	3 (toes)	5	Lost toes only
230	1	5	5 (toes)	6	Lost toes only
229	1	5	—	—	Lost 2 phalanges of 1 toe
231	1	5	5 (toes)	6	Lost toes only
243	0	3	4 (toes)	4	Lost toes only
244	0	3	3	4	Lost toes and 1 cm. of foot
245	0	3	3 (toes)	5	Lost distal 2 phalanges
246	0	3	3 (toes)	4	Lost toes only
15 Controls*			2 (9)† 3 (6)	4 (3) 5 (9) 6 (2) 7 (1)	Complete loss (11); loss of all but narrow plantar pad (4)

* These animals are the same as those used in a previous study (1).

† The numbers in parentheses indicate the number of animals.

tered daily by stomach tube as a 10 mgm. per ml. suspension in 1 per cent gelatin.

The extent of tissue loss was studied following controlled cold injury of the feet of rabbits in six cases in which rutin, 50 mgm. per kgm. daily, was administered both before and after injury, and in four additional cases in which rutin was administered only during the period after frostbite. The results are given in Table I. Observations on the development of gangrene and the extent of tissue loss in 15 control animals are included for comparison. In untreated animals three-minute immersion of the foot into a freezing mixture at -55°C . led to loss of the entire injured region in 11 of the animals and to loss of all but a small amount of tissue on the plantar surface of the foot in four animals. In none of the rutin-treated animals was the tissue loss as great as that observed in the controls. In most of the treated animals tissue loss was confined to the toes and in two instances involved only parts of the toes. The time of appearance of wet gangrene, although it occurred only in the toes of treated ani-

mals, was delayed as compared to the controls. The difference between the times required for the development of wet gangrene in the two series of animals was statistically significant ($P < 0.001$).

Doses of rutin similar to those used above failed to influence the course of cold injury to rabbit ears. Frostbite, produced by immersing the distal half of the ear in a freezing mixture at -55°C . for one minute, led to gangrene and complete loss of the injured part in 12 control animals. Five animals treated with rutin both before and after injury developed gangrene and lost all of the frostbitten parts of the ears. In one animal which received rutin the appearance of wet and dry gangrene was delayed one and three days, respectively, as compared to control animals. No delay was observed, however, when the experiment was later repeated on the remaining ear of the same animal.

Direct observation of the circulation (16) in the ear of a rabbit treated with rutin before frostbite showed that the local circulatory changes were similar to those seen in frostbitten rabbit ears following procaine block of the stellate ganglion (cf. section 4 below). Stasis, ordinarily complete in true capillaries within 10 minutes after thawing, was delayed up to 80 minutes in animals treated with rutin. Carbon particles were seen moving in large vessels and thoroughfare channels, but none was observed in true capillaries. Subsequently carbon accumulated in true capillaries during the growth of massive edema, but the amount was less than in untreated ears at the same time after frostbite.

Since the process of stasis following cold injury appears to depend upon the loss of plasma from damaged capillaries (3) the effect of rutin upon loss of trypan blue from the blood was studied. Adult rats, anesthetized with pentobarbital (37.5 mgm. per kgm. intraperitoneally), were subjected to cold injury by placing upon the shaved abdomen 5-ml. beakers, 18 mm. in diameter, filled with solid carbon dioxide. Seven animals were used as controls and five were given 200 mgm. of rutin per kgm. body weight by stomach tube one hour before injury. Two beakers were applied to each animal; one was left in place for one minute and one was left two minutes. Ten minutes after application of the beakers 0.5 ml. per kgm. of 1.5 per cent trypan blue was injected into

restricting the loss of tissue from gangrene following frostbite of rabbit feet. In 15 control animals, immersion of the foot to the level of the tuberosity on the fifth metatarsal in liquid at -55°C . resulted in complete loss of the exposed part in 11 cases and loss of all but a narrow portion of the plantar pad in four cases. Of ten animals similarly exposed but treated with rutin, nine lost only toes and one lost toes plus about 1 cm. of foot. Rutin was ineffective in preventing loss of tissue following frostbite of rabbit ears.

Rutin-treated animals did not develop stasis in the true capillaries of frostbitten ears as early as did untreated animals. The delay in onset of stasis was similar to that observed after procaine block of the stellate ganglion or rapid thawing of the frozen ear in water at $+42^{\circ}\text{C}$.

Rutin was found to delay the escape of trypan blue dye from the blood stream into frozen areas of abdominal skin in rats. The time from injection of the dye to first appearance of blue in the frostbitten areas of animals receiving rutin was approximately twice as long as that in controls. Vasoconstriction induced by intraperitoneal injection of Privine did not delay the appearance of trypan blue in frostbitten areas in spite of the marked blanching produced in normal skin areas, and the circulation time as measured by fluorescein was not increased by treatment with rutin. It therefore seems doubtful that rutin exerts its effect through peripheral vasoconstriction involving arterioles. Evidence is presented for the view that alteration in the pattern of blood flow through the capillary bed is a consequence of the administration of rutin.

Procaine block of the stellate ganglion delayed but did not prevent the development of gangrene in frostbitten ears.

The following drugs and hormones were found to be ineffective in the prevention of gangrene following frostbite.

1. Vasodilator agents: carbon dioxide, nitroglycerine and acetyl- β -methyl choline chloride (Mecholyl).

2. Anticoagulants: heparin and dicumarol.

3. Vasoconstrictors: epinephrine and/or sympathrine.

4. Steroid hormones: desoxycorticosterone and progesterone.

Alterations of blood volume, plasma colloidal

osmotic pressure and extracellular phase volume by the use of whole blood transfusion, administration of concentrated human plasma albumin and intravenous injection of sodium chloride solutions, all failed to prevent gangrene following standard cold injury.

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KIDNEY FUNCTION IN ADRENAL INSUFFICIENCY¹

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An association between adrenal insufficiency and disturbed kidney function has been demonstrated repeatedly. The first report on renal failure in Addison's disease was that of Smith (1) in 1897. In 1942 Talbott *et al.* (2) reviewed the literature and reported studies on patients who had adrenal insufficiency but were not in crises. They found formation of glomerular filtrate to be reduced. After institution of treatment with desoxycorticosterone acetate, there was an increase in glomerular filtration of 32 per cent in five patients. However, the values obtained were still below normal. With diminished absorption from implanted pellets of desoxycorticosterone acetate the rate of formation of glomerular filtrate decreased again. Moderate reduction of renal plasma flow was observed also as well as slight reduction of the maximum capacity of the tubules for excreting diodrast.

In recent years renotrophic effects have been demonstrated after the administration of various steroid hormones to mammals, especially androgens (3, 4). Testosterone propionate was found to increase the maximal capacity of the kidney tubules to excrete diodrast in castrated female dogs (5). No increase of renal function could be demonstrated when the same hormone was given to normal men, a eunuchoid male, and patients with kidney disease (6, 7), even when the dose was as large as 300 mg. per diem. Nevertheless, since the renotrophic action is believed to affect mainly the tubular cells and since in some cases of Addison's disease atrophy of these cells has been described (8 to 10) it was thought advisable to observe the influence of hormone therapy on the kidney by means of the more refined tests of renal function which are now available.

CLINICAL MATERIAL AND METHODS

Thirteen patients were studied. None had evidence of any primary renal disorder. None had proteinuria or abnormal urinary sediments when not in crisis. Most of the clinical data pertinent to the investigations of this report are summarized in Table I.

In the group with Addison's disease, there were four men and five women. All gave typical histories, namely, weakness, languor, anorexia, loss of weight, nausea, and vomiting. All had been in crisis on one or more occasions. Physical examination of each showed the characteristic pigmentation of the skin and mucous membranes. All developed hypotension and nausea when not treated. In each case the shape of the sugar tolerance curve after 50 grams of glucose by mouth was flat, with hypoglycemia developing two to three hours after administration of the glucose. In several of the earlier cases chloride excretion tests (11) had been attempted but could not be completed because of impending crisis. The neutral 17-ketosteroid excretion in the urine, done by the method of Talbot *et al.* (12), was decreased in all patients (Table I). Patient A. F. was found to have pulmonary tuberculosis which was considered quiescent after serial x-ray studies. In none of the other patients was there evidence of tuberculosis.

Four patients, two men and two women, had pituitary insufficiency. That of the men (W. K. and A. B.) was the result of chromophobe adenomata. The women, R. F. and A. M., had no evidence of tumors; in both the beginning of the disease followed pregnancy. They had the typical appearance of "pituitary myxedema" as described by Means *et al.* (13). In each of these four patients the excretion of neutral 17-ketosteroids in the urine was reduced to very low levels. Chloride excretion tests, done according to the regime outlined by Cutler, Power, and Wilder (11), gave results characteristic of adrenal insufficiency. Oral sugar tolerance curves were flat and there was failure of the blood sugar to rise when hypoglycemia was produced with the insulin tolerance test of Fraser, Albright and Smith (14). This test was not done in the case of A. M. because when she fasted for comparatively short periods her blood sugar frequently decreased to less than 50 mg. without tendency to rise unless carbohydrate was given.

A. B. revealed evidence by x-ray of a gradually expanding mass in the pituitary fossa since 1933. Definite symptoms of pituitary insufficiency had been present since 1935. Since 1940 he has gradually developed hyperten-

¹ The testosterone propionate and desoxycorticosterone acetate used in these studies were furnished by the Ciba Company.

TABLE I
Data concerning patients with Addison's disease and pituitary insufficiency

Patient	Sex	Age	Onset of symptoms	Treatment				Blood pressure	Blood			Urine neutral 17 K.S.	B.M.R.	Remarks
				Salt	D.C.A.*	Testosterone propionate	Adrenal cortex extract		Hgb.	Hemat.	Serum proteins			
				grams per diem	mg. per diem	mg. per week	ml. per diem	mm. Hg	grams per 100 ml.	per cent	grams per 100 ml.	mg. per diem		per cent
Addison's disease														
D. B.	F	36	1945-46	Oct. 5-10 '46	20			60-80/40-55	13.0		4.8	<0.5	-25	Had thyroidectomy in 1936 followed by hypothyroidism; taking thyroid 'till '46
				Oct. 11-20 '46	20	5		75-92/50-62						
				Oct. 21-28 '46	4-12	5		85-100/50-65	10.5	31	4.4			
				Oct. 29-Nov. '46	3-20			80-110/60-70	11.5	34	5.8			
				Dec. '46-Feb. '47	0	87.5	3-6†	84-102/64-64	8.0	24				
M. M.	F	39	1945	Feb. 7-10 '47	0	25.0	5	88-95/68-76	8.0	26	5.5		-41	Heart failure
				Feb. 11-Apr. 3 '47	18-8	25.0	5	88-110/60-80	8.0					
				Apr. 15-May 16 '47	8	25.0	5	85-120/64-80						
				Feb. '46	12			100-110/60-70	13.0	40	6.2	0		
				Feb.-June '46	3	2.5		95/60			5.1			
E. L.	F	41	1941	Aug. '46-Jan. '47	4 pellets	25.0		90-106/50-70	14.9	44			-18	Joint pains after D.C.A.
				Apr. '44-June '45	3			100-118/60-80	10.4-12.8		6.1	<0.5		
				June '45-Apr. '46	3	2.5		85-100/60-70	13.8	41				
				Apr. '46-June '46	6	2.5		90-100/58-62	13.0	38	5.5			
				June '46-Feb. '47	6	2.5	50	95-105/60-70		35				
E. W.	F	60	1913	Feb. 19-22 '47	3	2.5		100-115/65-80	12.2		5.7	<0.5	-2	Arthritis before onset Raynaud's phenomenon beginning Jan. '45
				May '44-Nov. '44	5			112/74			6.2			
				Nov. '44-Apr. '46	8 pellets	25.0		100-130/65-82	12.0					
				July '45-Apr. '46	4 pellets			95-100/70-75	12.6					

* D.C.A. = desoxycorticosterone acetate.

† Pork adrenal cortex extract in oil.

TABLE I—Continued

Patient	Sex	Age	Onset of symptoms	Treatment				Blood pressure	Blood			Urine neutral 17 K.S.	B.M.R.	Remarks
				Salt	D.C.A.*	Testosterone propionate	Adrenal cortex extract		Hgb.	Hemat.	Serum proteins			
				grams per diem	mg. per diem	mg. per diem	ml. per diem	mm. Hg	grams per 100 ml.	per cent	grams per 100 ml.	mg. per diem		per cent
Addison's disease—Continued														
S. E.	F	48	1943	3	1.25	17.5		98-130/62-90 84-118/64-68 116/66 115-125/75-82	11.0-13.0 11.1 11.1	34 39	5.5	<0.5	+14	Hypertension before onset Rheumatoid arthritis before onset Increased joint pains after D.C.A.
J. M.	M	45	1944	3-6	5 pellets			112-126/60-75	12.0 10.5-12.0	35 31-35	5.4 4.8-5.5	6.4	-7	
H. W.	M	30	1943	3-6	5 pellets		occasional	112-126/60-70 98-110/62-76	14.7 14.0	42	6.0 5.8	4.3	-17	Raynaud's phenomenon before onset
A. F.	M	44	1943		9 pellets 4 pellets			92-120/56-84 110-122/70-80	14.0 13.0		5.5 5.7	3.3	-16 -20	
T. D.	M	52	1943	3	7 pellets 4-5			122-128/78-80 150-170/94-100 100-115/65-80 132-170/90-100	13.8 12.8 13.3	33 36 38	5.4 5.0	4.4 3.8	-7	Hypertension beginning Feb. '45 Raynaud's phenomenon
Pituitary insufficiency														
R. F.	F	47	1939	6	1.25			100-115/75-80 100/68 94-106/64-75 88-115/58-70 100-110/60	9.4 12.7 10.0-11.0 10.0 13.0	30-32 38	5.5	0.8	-33 -39 -14	Thyroid 0.065 gram/day No medication Thyroid 0.100 gram/day
A. M.	F	47	1931	3				80-88/60-68	9.3	28	4.8	0	-44	Frequent hypoglycemia
W. K.	M	31	1944	3-6		occasional		90-115/60-75	10.5-13.0	31-37		3.1	-26	No testosterone propionate 6 months before test
A. B.	M	65	1935	3-6 6				85-110/58-70 140-210/100-110	11.8-12.0 11.6		5.4 5.3	0.5	-30	Hypertension beginning 1940

TABLE II
Renal clearance studies in Addison's disease and in pituitary insufficiency

Patient	Sex	Date	Glomerular filtration rate ml./1.73 sq.m./min.	Effective renal plasma flow ml./1.73 sq.m./min.	Effective renal blood flow ml./1.73 sq.m./min.	Maximum tubular excretion mg. PAH/ 1.73 sq.m./min.	Filtration fraction (GFR/RPF) per cent	RPF/Tm	RBF/Tm	Blood pressure mm. Hg	Urine flow ml./min.	Remarks
Addison's disease												
D. B.	F	10-11-46 10-28-46 2-11-47	76 71 85	485 481 424	700 560	53 53 47	15.8 14.7 20.0	9.1 9.1 9.0	13.2 11.9	80/40 85/50 109/75	4.00 1.70 2.00	Salt therapy * Salt, D.C.A. 17 days, pork adrenal extract 8 days Aqueous adrenal cortex extract 50 ml. daily—4 days
M. M.	F	2-15-46 9-30-46	63 64	390 300	650 536	52	16.2 21.2	7.5 5.8	12.5 10.3	98/70 104/60	6.94 1.75	Salt Salt and D.C.A.
E. L.	F	6-12-46 12-16-46 2-22-47	51 56 66	274 212 259	465 342 400	53 48 52	18.7 26.5 25.5	5.2 4.4 5.0	8.8 7.2 7.7	92/60 100/80 96/70	1.48 1.53 1.80	Salt and D.C.A. Salt and D.C.A. and testosterone propionate Salt and D.C.A. plus 50 ml. aqueous adrenal cortex extract—4 days
E. W.	F	4-15-46	56	225	358	52	24.8	4.3	6.9	100/70	2.89	Salt, D.C.A., and testosterone propionate
S. E.	F	1-22-46 6-24-46 12-9-46	61 68 60	241 267 244	365 400	40 49 48	24.6 25.5 24.6	6.0 5.5 5.0	9.1 8.3	130/78 130/86 130/88	2.72 2.00 1.70	Salt and D.C.A. Salt, D.C.A., and testosterone propionate Salt, D.C.A., and testosterone propionate
J. M.	M	2-11-46 12-4-46	91 97	616 620	893 960	91 78	13.1 15.6	5.6 8.0	9.8 12.3	110/60 120/80	2.48 1.95	4 months after implantation of D.C.A. pellets 14 months after implantation of D.C.A. pellets, extra salt
H. W.	M	5-23-46	69	390	700	88	17.9	4.4	8.0	105/60	1.74	12 months after implantation of D.C.A. pellets, extra salt
A. F.	M	3-25-46 4-29-47	80 67	370 319	463	67 66	21.7 21.0	5.5 4.8	7.0	110/84 90/70	3.79 2.00	12 months after implantation of D.C.A. pellets 25 months after implantation of D.C.A. pellets, extra salt
T. D.	M	5-8-46	67	320	516	48	20.9	6.7	10.7	170/100	3.89	D.C.A.
Pituitary insufficiency												
R. P.	F	12-5-45 1-2-46 1-27-46 4-22-46 9-6-46 1-23-47	77 73 84 78 84 69	273 322 340 276 261 251	400 457 482 392 405	51 51 48 51 56 44	28.2 22.6 24.6 28.2 32.3 27.2	5.2 6.3 7.1 5.5 4.7 5.7	7.9 8.9 10.0 7.7 9.2	110/70 100/60 105/72 110/60 150/96 126/88	6.15 4.33 4.39 5.61 8.99 7.85	Salt therapy Thyroid plus salt D.C.A. plus salt Testosterone propionate plus salt Testosterone propionate plus salt Testosterone propionate plus salt
A. M.	F	6-25-47	76	255	354	36	29.8	7.0	10.0	88/60	3.60	Salt therapy
W. K.	M	1-15-46 2-5-46	76 72	474 589	718 890	82	16.0 12.2	7.2	10.7	100/75 120/80	1.95 9.65	Salt therapy Salt therapy
A. H.	M	10-16-46	54	250	378	34	21.7	7.2	11.0	170/100	3.97	Salt therapy
Average values—female—normal												
Average values—male—normal (16, 19, 20)												

* For duration and amount, see Table I.

sion, yet tests enumerated above have continued to be abnormal and he develops symptoms of sodium chloride deficiency when not treated with salt or testosterone propionate.

All the patients were studied when in good clinical condition, it being our purpose to avoid the complicating factors present in crisis or shock. Observations were made while the patients were under basal conditions, *i.e.*, about 14 hours after the previous meal. Several patients, who had a tendency to develop spontaneous hypoglycemia, were given 200 ml. of orange juice one hour before the test since it was found that this did not change the results.

Two patients with Addison's disease (D. B. and M. M.) and all those with hypopituitarism were studied first on salt alone. The other seven patients with Addison's disease had been treated with desoxycorticosterone acetate (D.C.A.) before studies were begun. After satisfactory observations had been made under either of the above conditions, treatment with other hormone preparations was superimposed when possible. We were thus able to study the influence of desoxycorticosterone acetate (D.C.A.), testosterone propionate, aqueous adrenal cortex extract, extract of pork adrenal cortex in oil, and desiccated thyroid, U.S.P.

Renal function studies were made by the multiple period technique advocated by Goldring *et al.* (15). Rates of glomerular filtration were determined as mannitol clearances (16). Effective renal plasma flow and maximum tubular excretory capacity were determined from para-amino-hippuric acid (P.A.H.) clearances at the proper plasma levels as outlined by Chasis *et al.* (17). Analyses were made upon plasma filtrates prepared according to Fujita and Iwatake (18) and on diluted aliquots of urine. Mannitol was determined according to the method of Smith, Finkelstein, and Smith (16). Para-amino-hippuric acid (P.A.H.) was determined according to the method of Bratton and Marshall as modified by Smith *et al.* (19). All results were corrected in proportion to surface area, the final figure being in terms of a surface area of 1.73 sq. meters. Values for these clearances obtained in our laboratory on normal persons are approximately the same as those published by Smith, Goldring, Chasis *et al.* (17, 20, 21).

RESULTS

The results of all observations are recorded in Table II, and a typical test for each patient when either on sodium chloride alone or when receiving D.C.A. is illustrated in Figure 1.

Rates of glomerular filtration were below normal in all patients at all times. This is in agreement with the data published by Talbott *et al.* (2). Treatment with hormones resulted in little if any improvement above that found when salt was used in sufficient amounts to cause adequate improvement in the clinical condition and restoration of

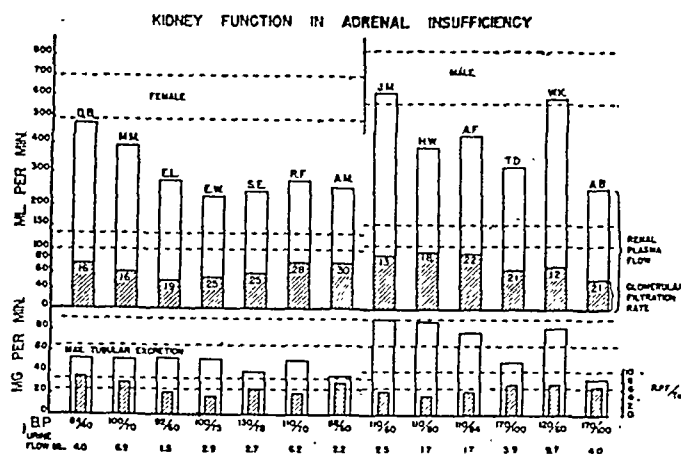


FIG. 1. RENAL CLEARANCE STUDIES IN ADDISON'S DISEASE AND IN PITUITARY INSUFFICIENCY

Patients, D. B., M. M., R. F., A. M., W. K., and A. B., were receiving sodium chloride in sufficient amounts to control symptoms, serum electrolytes, etc. All other patients were receiving desoxycorticosterone acetate. Normal values for the various functions fall between the broken lines. The maximum tubular excretion is for P.A.H. The numbers appearing in the upper, cross-hatched columns are the filtration fractions.

extracellular electrolytes, water and blood pressure (see D. B., M. M., and R. F., Table II). In A. F. there was a slight decrease between the observations made 12 months and 25 months after implantation of nine pellets of D.C.A.² Decreased electrolyte retention between the two periods of observation was to be expected and can explain the decrease in glomerular filtration.

When extract of adrenal cortex was given in large amounts (50 ml. daily) there was perhaps slight increase of filtration but the values were still far below normal.

The filtration fraction

$$\left(\frac{\text{Glomerular Filtration Rate}}{\text{Renal Plasma Flow}} \right)$$

varied considerably at different times in the same subject and also from individual to individual. In general it was normal or high but in three instances it was exceptionally low. The variations cannot be explained without more control observations prior to the institution of hormonal therapy. In the two female patients with Addison's disease (D. B. and M. M.) who had previously been controlled with salt, the filtration

² Two months after the last observation reported here the remnants of two of the nine pellets implanted 27 months previously were recovered.

fraction increased after administration of adrenal cortical hormones which suggests that specific medication may have been a contributing factor.

The maximum excretory capacity of the tubular cells for para-amino hippuric acid (P.A.H.) showed the most interesting deviations from normal. It was low in all female patients and normal in all male patients with the exception of the two men, T. D. and A. B., who had hypertension. As both had had elevated blood pressures for more than a year, the decreased excretion of P.A.H. may have been a hypertensive phenomenon (19). In Patient T. D., hyperpiesia followed excessive treatment with desoxycorticosterone. In A. B. the etiology was not clear and it was present in spite of evidence of insufficiency of the anterior lobe of the pituitary and the adrenal cortex.

Testosterone propionate was given intramuscularly to several of the female patients (see Table I). S. E. received 25 mg. every other day for 20 weeks. At the end of this time a second series of observations showed no significant change from the controls. Treatment was then continued with 25 mg. per week for 24 weeks and again there was no improvement. Patient D. B. was given 25 mg. every other day for four weeks and then 25 mg. once a week without influencing the function studies. To test the possibility of causing improvement by giving small doses over a long period of time, Patient R. F. was given 25 mg. weekly for over a year and E. L. the same dose for six and a half months but no change was observed in either case.

Admittedly the doses given the last two patients were rather small but it was enough to cause marked improvement in their clinical condition. The amount of hormone given S. E. on the other hand should have been ample to bring out any potential effects.

Aqueous adrenal cortex extract was given to patients, D. B. and E. L., in doses of 50 ml. daily for three days, followed by administration of the extract during the tests, and no change was observed in the Tm for P.A.H. D. B. had received 5 ml. of extract daily for three months before the large amounts were given.

Thyroid substance likewise produced no change when given to one female patient, R. F., who had pituitary myxedema.

Effective renal blood flow was always reduced below normal. This was also true of the effective renal plasma flow except in two male patients who had moderate anemia (J. M. and W. K.). In the latter the effective renal plasma flow was within low normal values but the whole blood flow was well below normal. Similar findings have been reported in various anemias by Bradley and Bradley (22). Whether the patients were controlled on salt alone or receiving specific therapy seems to have made little if any difference in this function.

DISCUSSION

The distinctive features of the derangement of renal function found in these studies were (a) permanent decrease of renal blood flow and glomerular filtration rate in all patients and (b) reduction of the maximum capacity of the tubules to excrete P.A.H. in all female patients. The consistent finding of decrease of renal blood flow and glomerular filtration rate regardless of the presence or absence of impairment on the part of the tubular cells to excrete P.A.H. is a point of considerable interest. The height of the blood pressure did not seem to be a factor as long as the patients were in good clinical condition. Indeed, comparatively high functional values were obtained at times even in the presence of low blood pressure (see D. B.). Hydration of the patients was always good so that decreased volume of the circulating blood was probably not responsible. While this has been found below normal in untreated adrenal insufficiency it is restored to normal by treatment with desoxycorticosterone or adrenal cortex extract (23, 24). As may be noted in Table II, low renal blood flow and glomerular filtration rates were obtained in our patients while receiving adequate treatment with these preparations. The several tests employed may indicate the status of various parts of the renal vasculature. Consideration of all the data compels us to postulate a reduction in the effective vascular bed in the kidneys with resulting decrease of renal plasma flow and concomitant reduction in the rate of glomerular filtration.

In those patients with Addison's disease whom we were able to study while on salt therapy alone, the ratios, R. B. F./Tm, were low normal and this fact may be interpreted as evidence against rela-

tive ischemia of the functioning excretory tubular tissue of the kidneys (21). The Addisonian patients who had been under the influence of desoxycorticosterone acetate for long periods of time as well as all those with pituitary insufficiency were found to have a relatively greater reduction of renal blood flow than of their maximum ability to excrete P.A.H. (low ratio of R.B.F./Tm) or relative ischemia of the tubular tissues (21). It is uncertain whether this latter finding was the result of disease or was due to treatment.

Information on whether specific therapy affects the filtration apparatus should be afforded by examination of the filtration fraction. Talbott (2) found low filtration fractions in two cases of Addison's disease treated with sodium chloride alone, and this agrees with our observations in two cases studied under similar conditions. All but one of our nine Addisonian patients had normal or slightly elevated filtration fractions after treatment with D.C.A. or aqueous adrenal extract. Of four pan-hypopituitary patients in our series all but one had high filtration fractions. This was true also in two cases studied by Talbott. From the evidence at hand it appears that the patient with Addison's disease prior to specific treatment will show a low filtration fraction in contradistinction to pan-hypopituitarism where the untreated subject may be expected to have a high filtration fraction.

Unusual sensitivity of the blood vessels in patients with Addison's disease to vasoconstrictor as well as vasodilator influences has been reported (25, 26). Roth, Robinson and Wilder (25) found that the unusual hyperreactivity of the Addisonian patient to the constrictor influence of cold was increased by treatment with desoxycorticosterone acetate. From the data available it seems more than probable that treatment with this steroid exerted a vasoconstrictor influence on the vascular bed of the kidneys in our patients. The unexplainable variations in general obtained in some of our patients, especially those with pan-hypopituitarism, are probably due to opposing influences and trophic factors which are not understood.

The failure of any of the functions to improve with the medication given may have been due to several causes. The dose of adrenal cortex hormone may have been insufficient or not given for a

long enough time, in spite of the prompt and adequate restoration which such doses cause in other functions (27, 28). Irreversible structural or functional changes may have taken place. Whether or not histological changes accompany functional changes in kidneys in adrenal insufficiency has been the subject of considerable controversy (8 to 10, 29, 30). It is possible that this may be clarified to some extent if the sex of the patients or animals studied is taken into consideration. Gaunt (31) was unable to correct the defect in water diuresis in adrenalectomized rats, even when adequate hormonal therapy was instituted in less than a week after adrenalectomy.

Finally, the factors responsible for maintenance of the functions mentioned may not have been present in any of the preparations given. The failure of testosterone propionate to restore the Tm/P.A.H. in the female patients is of special interest. The hormonal factors which help maintain this function in the males are undoubtedly made by the testis, but cannot be replaced by testosterone propionate.

SUMMARY

1. Studies on glomerular filtration rate, renal plasma flow and maximum excretory capacity are reported on 13 patients with adrenal insufficiency.
2. Maximum excretory capacity was reduced in female patients and was not affected by hormone therapy.
3. Filtration rates were reduced and it is postulated that absolute reduction at least in the long standing, well controlled Addisonian, is secondary to reduction in renal plasma flow.
4. Changes in the renal blood flow are discussed with regard to the disease per se and to hormonal therapy.

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STUDIES ON PAIN: AN INVESTIGATION OF SOME QUANTITATIVE ASPECTS OF THE DOL SCALE OF PAIN INTENSITY

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Wolff and his collaborators (1) have, for a number of years, reported estimates of intensity of pain in terms of one to ten "plus," one plus representing a minimal pain and ten plus the worst pain ever experienced by the subject. This method was valuable as a means of roughly estimating relative pain intensities, especially in studies of experimental headache. Others have resorted to procedures such as squeezing the biceps muscle or placing pressure on the styloid process (2), to produce pain of high intensity which the patient was asked to compare with his spontaneously occurring pain. Although the estimates of pain intensity based upon these procedures have been useful, the stimuli have lacked measurable aspects, and, therefore, have not been suitable for a quantitative study of pain intensity. In general, methods of estimating the intensity of painful sensations have suffered from two principal defects. First, it has been difficult for the investigator to separate from the entire pain experience the property of a painful sensation which is *intensity*. Indeed, at least one thoroughgoing investigator was led to the conclusion that the difficulties arising in this respect did not permit reliable estimates of pain intensity to be made (3). Second, the study of pain has been inadequate as regards the intensity aspect of the sensation, and no common basis has been provided for intercomparison of experimental data obtained under various circumstances in different laboratories.

In a previous communication (4) a scale of pain intensity was proposed, based upon the ability of normal individuals to discriminate differences in intensity of painful sensations. The unit of this scale was chosen arbitrarily as the sum of two just noticeable differences, and the term "dol" was suggested as the name of the unit of pain intensity. Three important questions concerning the dol scale are: First, does the sum of the two just noticeable differences represent the same difference

in pain intensity in all parts of the dol scale? Second, are the numbers representing the pain intensity in dols associative, that is, is a four-dol pain twice as intense as a two-dol pain, etc.? And third, is the scale sufficiently representative of common experience to serve as a basis for intercomparison of data? Reported herein is evidence indicating that the above questions should be answered affirmatively.

The dol scale was first established when, using a three-second exposure of radiant heat on the skin as the stimulus, the difference in intensity of radiation which elicited a just noticeable difference in pain sensation (jnd) was measured. Measurements were begun with the pain threshold stimulus (220 mc/sec/cm²) and carried to progressively greater stimulus intensities. As the intensity of radiant heat was increased beyond that causing tissue damage, greater increments of stimulus were required to elicit distinguishable differences in pain intensity. For example, the pain elicited by a stimulus of 480 mc/sec/cm² could barely be differentiated from that elicited by 680 mc/sec/cm², and greater amounts of stimulus elicited no distinguishable increase in pain intensity. Between the threshold stimulus and the maximum stimulus which could be discriminated, there were found to be 21 just noticeable differences. The dol was defined as the difference in pain sensation evoked by stimuli differing in intensity by the sum of two jnd's. The 21 jnd's between the threshold sensation and the "ceiling" pain were thus equivalent to ten and one-half dols. It should be emphasized that painfulness, measured in dols, refers only to the intensity aspect of pain sensation and is estimated from the intensity of the stimulus which is the directly measurable quantity (millicalories per second per square centimeter).

It was recognized that the number of millicalories per second per square centimeter required

to produce a one-dol change in pain intensity increased with the intensity of the stimulus. However, it was hoped that the size of the dol would be the same in terms of painfulness throughout the scale, in as much as it might be logically assumed that a just noticeable difference in painfulness would be the same for a mild pain as for a severe pain. Indeed, this was Fechner's argument when, in the last century, he made his well-known modification of the Weber Law (5). For example, recent studies of hearing (6) show that for loudness, the intensity attribute of hearing, the sum of the jnd's does not constitute a uniform sensory scale. That is, the scale of loudness based on the sum of jnd's does not coincide with the scale derived from estimating the relative intensity of sounds in terms of a sound of fixed intensity. It is, therefore, evident that in the case of loudness at least, Fechner's assumption that a jnd is of the same sensory magnitude regardless of the intensity of sensation is not valid, and that the jnd for loudness is smaller for low intensities of sound and larger for the high intensities of sound.

The technique of comparing the intensity of one sensation with another has been termed by the psychologists "fractionation" and this consists of requiring the subject to estimate the intensity of a sensation in terms of fractions or multiples of a standard, fixed intensity. It was desirable, therefore, to investigate pain by means of the fractionation methods and to ascertain whether or not the size of the dol was uniform throughout the scale of painfulness.

It is the purpose of this communication to report the results of studies in the estimation of painfulness by means of fractionation, and to compare this method of estimating pain intensity with the scale of pain set up on the basis of the jnd's.

METHOD

The painful sensation studied in these experiments was produced in the skin by a three-second exposure to an intense thermal radiation. The apparatus used to heat the skin has been previously described (4) and mention is made here only of the necessity of checking the instrument's calibration at frequent intervals with a standardized radiometer. Our practice has been to calibrate the instrument before each experiment, and data so obtained show that, as the heat lamp ages, its radiant heat output decreases for any given reading of the voltmeter.

This effect may possibly be due to the gradually increasing opacity of the glass bulb as the result of distillation of metal from the incandescent filament.

In one series of experiments the authors served as both experimental subjects and observers; and in a second series 70 medical students conducted carefully supervised experiments on each other, acting in turn as subject and observer.

Fractionation of the painful sensation was accomplished in the following manner. Each subject was exposed at the beginning of an experiment to a standard stimulus evoking a pain which on the scale of just noticeable differences corresponded to eight dols. Subjects were then exposed to eight intensities of stimulus chosen to evoke pain intensities of 1, 2, 3, . . . 8 dols. Three stimuli at each of the eight intensity levels were given in random order. The subjects were asked to report the intensity of the pains in terms of fractions of the initial, standard pain. As a rule, the standard stimulus was repeated once during the course of the experiment to refresh the subject's memory. Care was taken not to overstimulate an area of the skin, as the skin becomes hypersensitive as the result of too rapid stimulation. An interval of at least five minutes is desirable between stimuli.

RESULTS

The pain intensities reported in Series 1 are shown in Figure 1. This represents 72 tests on three subjects. The scatter of the reports is about one dol on either side of the average report for a particular stimulus intensity, and reports as much as one and one-half or two dols from this average were not uncommon. As the experiments proceeded it became apparent that the experience

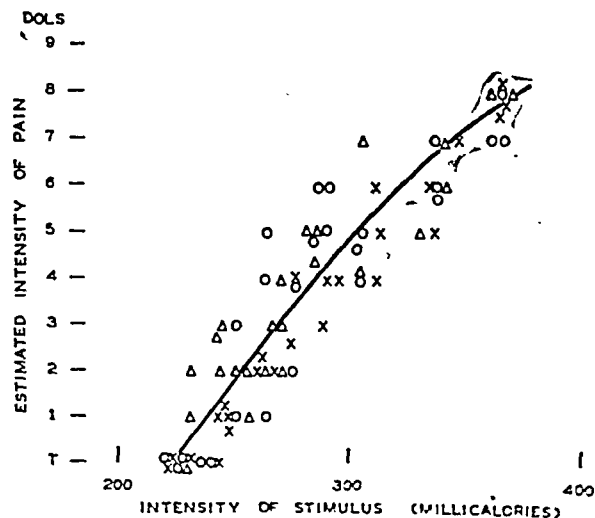


FIG. 1. ESTIMATES OF PAIN INTENSITY AS FRACTIONS OF A PAIN OF EIGHT DOLS INTENSITY

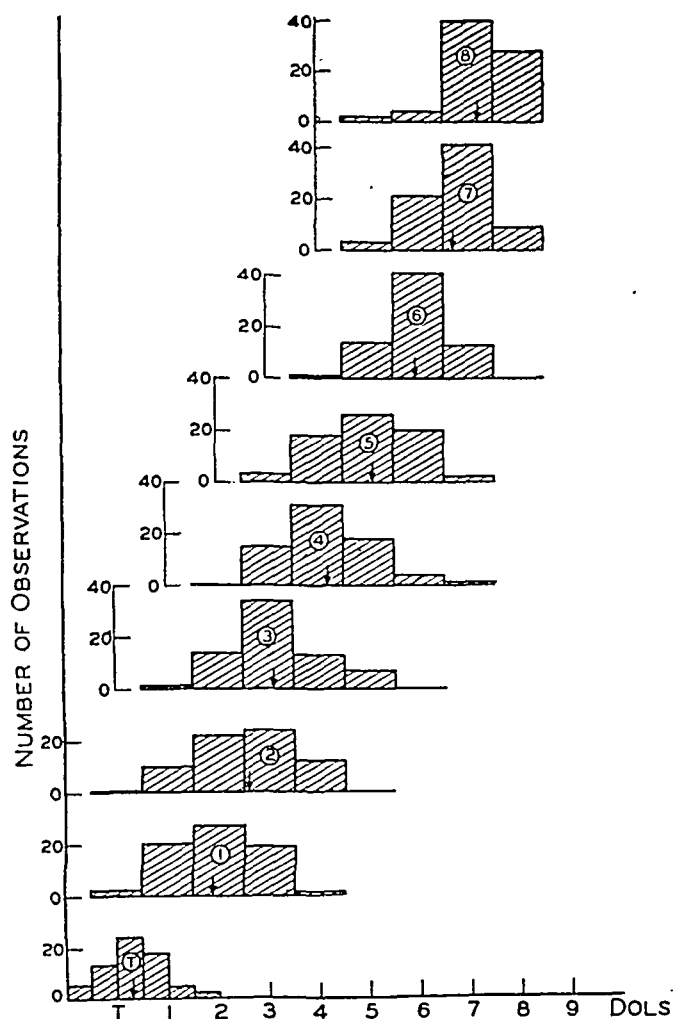


FIG. 2. DISTRIBUTION DIAGRAMS OF REPORTS OF 70 MEDICAL STUDENTS REPORTING PAIN INTENSITIES AS FRACTIONS OF EIGHT DOLS

of the subjects, as indicated by variations in reports, did not increase the accuracy of reporting. Lack of concentration upon the procedure was the most important single factor causing variability in reports. Memory was not a significant factor because the accuracy of reporting showed no change when in the course of the experiment the standard was presented repeatedly. The heavy line in the figure represents the dol scale based on measurements of the just noticeable differences, and this line coincides exactly with the average of the reports for all the stimuli given (see also Figure 4).

The second group of experiments, done by the medical students under supervision, indicates the scatter of reports from untrained but intelligent subjects and observers. The students followed the procedure outlined above and the results are

shown in Figure 2 as a series of scattergrams. In general, the average of the reports of the intensity of pain evoked by each unknown stimulus was within one dol of the value determined by the method of just noticeable differences and the scatter of the reports was approximately the same as that obtained with experienced observers.

These results may be compared with those of experiments done in this laboratory prior to the evolution of a scale of painfulness, because, in the course of several years' experimentation with the thermal stimulus for producing pain, reports were made from time to time on the intensity of pain elicited by various intensities of thermal radiation. In these reports as well as in those for estimating headache (2), intestinal pain (7), pain from immersion of an extremity in cold water (8), etc., it has been the practice for subjects to make reports of pain intensity in "pluses," using an arbitrary scale of one to ten plus to represent the intensities of pain between threshold and the most intense pain experienced. This procedure is essentially fractionation based on the individual's life experience. The results of 45 such estimates of pain intensity made over a period of several months by three subjects are shown in Figure 3.

It will be noticed that although ten plus was considered to be the most intense pain ever experienced, reports of 11 + and 12 + were made

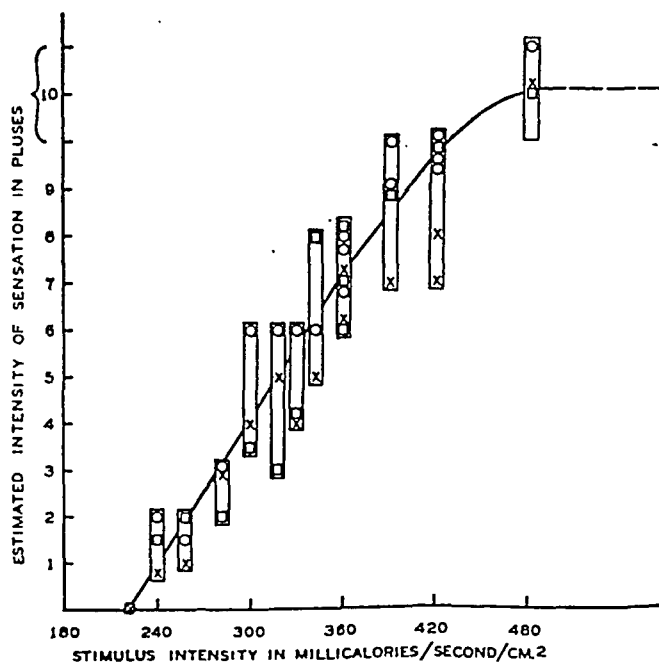


FIG. 3. ESTIMATES OF PAIN INTENSITY IN TERMS OF THE MOST INTENSE PAIN EVER EXPERIENCED

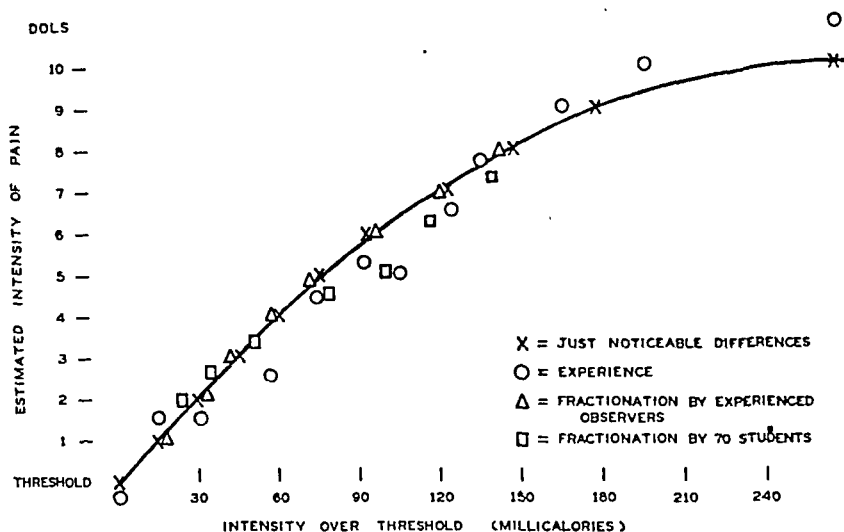


FIG. 4. A COMPARISON OF METHODS OF ESTIMATING PAINFULNESS

(a) (X) Integration of the just noticeable differences. (b) (□, Δ) Fractionation of an eight-dol pain by 70 medical students and three experienced observers. (c) (○) Fractionation of the most intense pain experienced.

for the most intense stimuli. This was due to the lack, at that time, of any concrete standard pain with which to compare the painfulness of these intense stimuli, and the subjects were consciously "stretching" the imaginary scale as it was evident to them that they could experience more degrees of painfulness than they had imagined. The solid line drawn in Figure 3 again refers to the scale of pain previously suggested from studies of the just noticeable differences, equating one "dol" to one "plus" value. The agreement between the dol scale and the "plus" values is close except at the highest intensities.

A summary of the various methods of estimating the intensity of the experimental pain is shown in Figure 4. The heavy line is drawn through the points determined by adding the jnd's. The results obtained with the three experienced observers coincide with this line and the observations made by the students are near the line although with more deviation. The judgments from the plus scale are included to illustrate the fact that these judgments based on the life experience of the subject are in close agreement with the dol scale of pain.

A third series of experiments was designed to ascertain the effect of fatigue, minor mood changes and feelings of general effectiveness upon the perception of pain. It had been previously demon-

strated that these variables did not affect pain threshold measurements (9). Three subjects were studied almost daily over a period of six weeks in the following manner. At about noon each day the subjects reported to the laboratory for testing. A record was made of the mood, feeling of effectiveness, and of any unusual activity or experience in the subject's life during the past 24 hours. Following this record, pain thresholds were measured and three unknown stimuli were given. A standard stimulus was not presented for comparative purposes in these experiments. The subject made a report of the intensity of the pain evoked. The unknown stimuli were distributed at random between the threshold stimulus and that causing a nine-dol pain. The ten-dol stimulus was avoided because it produces tissue damage.

The results of this study are shown in Figure 5 in terms of deviations from the report expected on the basis of the dol scale. The deviations are plotted as positive or negative according to whether the reports were greater or less than the dol scale values. Each point is the average of the three reports of the subject for the day in question. The average deviation of the reports from the dol scale is minus one-half dol, that is, the subjects underestimated rather than overestimated the intensity of the stimuli. The maximum deviation of

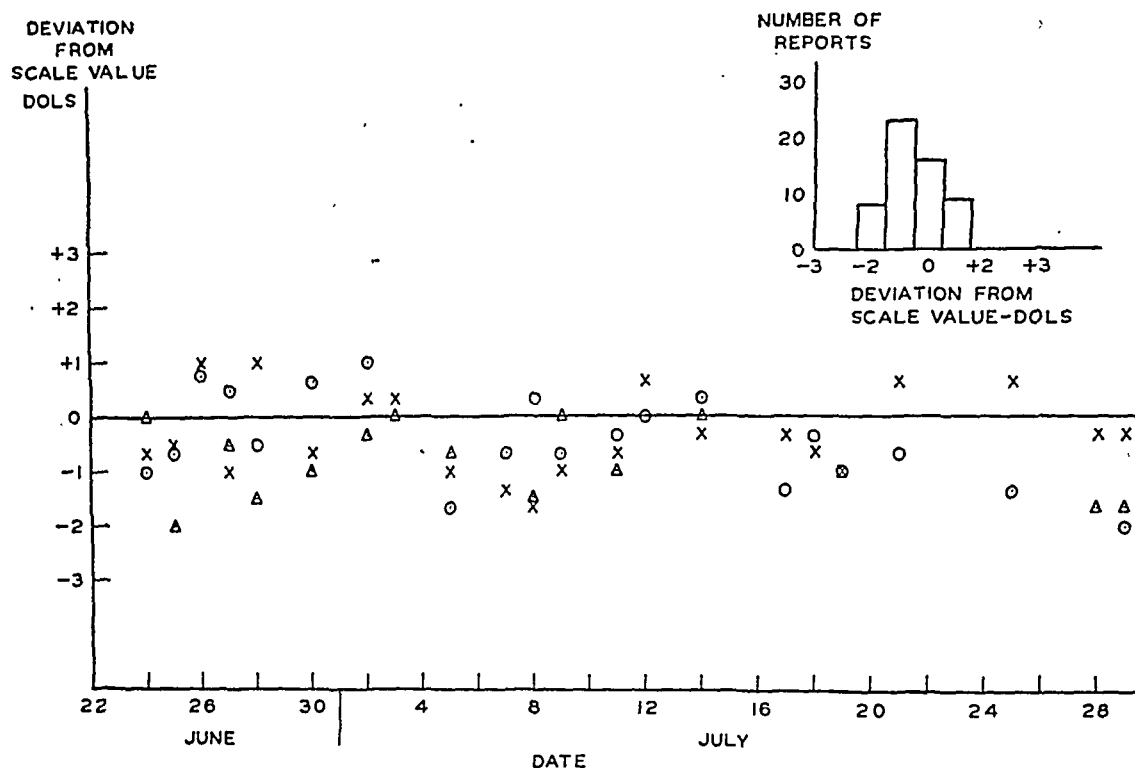


FIG. 5. DAY TO DAY VARIATIONS IN REPORTING PAIN INTENSITY BY THREE SUBJECTS

the reports from the scale value was two dols and the mean deviation plus-minus one-half dol. Estimates of mood and of general effectiveness in the three subjects varied from day to day. Fatigue resulting from 30 hours of wakefulness was encountered in one subject, and severe disappointment in another. Tenseness, mild depression, exhilaration, contentment were reported on different days. These mood changes and fatigue did not appear to be associated with any predictable change in the ability of the subject to estimate pain intensity. This result agrees with the observations previously made on the pain threshold (9).

DISCUSSION

The data presented in Figures 1 and 2 indicate that there is a variation of plus-minus 10 per cent in a single measurement of pain intensity obtained by this method. The accuracy of estimation does not depend upon the subject's experience with the method and it is possible that pressing a subject too hard for accurate reports will lead to a greater scatter in the results than is otherwise obtained.

Without a standard pain for comparison pain intensities at high levels are difficult to estimate, as shown in Figure 3. Furthermore, it is important to call the subject's attention to the fact that it is

the intensity aspect of the sensation which is being estimated. The overall distress of the individual, in which the uncertainty as to duration of the pain, the amount of tissue involved and the implications of the situation are of major importance, should not be confused with intensity of pain.

It is shown in Figure 4 that the scales of painfulness, as determined by fractionation of intensity, and by integration of the just noticeable differences, coincide. This fact implies that for cutaneous pain sense, the sensory magnitude of jnd's is the same in all parts of the dol scale. That is, increasing the painfulness by a jnd at one dol causes the same change in sensation as increasing the painfulness by the same amount at eight dols. For example, although this type of relationship does not hold for the loudness of sound, it does hold true for the perception of the pitch of sound, keeping the loudness constant. The fact that the pain scales coincide would *a priori* make it seem likely that judgments of pain intensity, whether made on the basis of increments of increasing intensity or in fractions of a "top" pain, would lead to the same results. It is, therefore, not surprising that estimates of pain intensity in terms of "pluses" as used by Wolff and his associates agree in relative value with the dol scale, in

spite of individual differences in the methods by which the observers may have arrived at their estimations of pain intensity. The numbers of the pain scale which represent the intensity aspect of pain are capable of being added and divided according to the ordinary rules of arithmetic, and it is possible to define one dol both as the sum of two jnd's and as one-tenth of the intensity of the ceiling pain. It is proper, also, to speak of one pain as being either "twice as strong" as another or as equivalent in intensity to the sum of two smaller pains. This suggests that there is only one scale of pain intensity and that the estimates by normal subjects on the basis of a standard pain will result in reports which can always be evaluated in terms of the dol scale.

The agreement of the estimations of pain intensity from general experience with the more carefully determined dol scale is of significance. It lends validity to the use of such a scale of "pluses" as a quantitative procedure in past experiments on headache, etc. Also, it implies that the clinician's interpretation of the patient's estimates of pain in terms of "mild," "moderate," "severe" and "extreme" represents fairly reproducible divisions of the intensity scale of pain. Thus, a "mild" pain is probably one that has an intensity between threshold and two dols, a pain such as is usually abolished by the use of acetylsalicylic acid or some other coal tar derivative. A "moderate" pain is one of intensity between three and six dols, and is a pain that cannot be tolerated for any length of time without loss of sleep and disturbing reactions. Codeine and other opiates are usually required to manage a pain of this intensity. A "severe" pain has an intensity between six and nine dols and maximal amounts of the most effective analgesics are required to reduce such pain to a threshold level (10). An "extreme" pain, nine to ten and one-half dols, will require complete anesthesia, either general or locally applied to the source of pain, to bring relief.

A further point of interest in this connection is the accuracy with which judgments can be made of the intensity of a pain from memory of one's general experience. It is as if a normal adult had in his mind at all times a keen sense of the intensity aspect of pain, so that a person without experience in making judgments and discriminations

of this sort can give a surprisingly accurate estimate of the intensity of a pain. Pain memory is apparently very good, and is undoubtedly important to the body economy.

Over-reaction to pain, due to anxiety and fixed attitudes toward pain sensation was encountered in four of the 70 medical students. These at first exhibited an almost complete lack of discriminatory ability during the observations, often reporting a one-dol pain as six dols and vice versa. Also; pain threshold measurements were difficult to make on these individuals. It was apparent that the attitude of these subjects toward the experiment and their fellow student observers prevented their making proper estimations. These four subjects were studied individually by the authors following their class experiments, and, after several trials, normal reports were usually obtained. Two of these four subjects had suffered major physical injuries and had experienced pain over long periods of time. Similar difficulties in estimating pain intensities, and in recognizing the pain threshold, were sometimes encountered in patients. However, with reassurance and repeated trials, reproducible results were obtained.

SUMMARY AND CONCLUSIONS

1. Using a three-second exposure of thermal radiation on the skin as a painful stimulus, measurements have been made of the stimulus intensities which evoked painful sensations of various relative magnitudes. Three series of experiments were performed, in the first of which three experienced observers reported the relative intensities of pain in terms of fractions of an eight-dol pain. In the second series of experiments 70 medical students were similarly studied. In a third series of experiments the effects of fatigue and minor mood changes upon discriminations of relative intensity of pain were studied.

2. It has been ascertained that the scale of painfulness based on estimations of pain intensity as fractions of a known pain, coincides with the scale based on the summing of just noticeable differences in pain sensation. The dol as a unit of painfulness can thus be defined as the sum of two jnd's in pain sensation, or as approximately one-tenth the intensity of the ceiling pain.

3. The accuracy of estimating pain intensity is limited by the ability of the individual to discrimi-

nate differences in pain intensity. This limit is plus-minus one-half dol; the spread of reports about a mean value was approximately plus-minus one dol. Experience in reporting pain intensities did not increase the accuracy of estimation.

4. Moderate fatigue and day to day variations in mood were not associated with an appreciable change in the ability to estimate pain intensity in three normal subjects tested over a period of six weeks.

5. From the above studies it is concluded that the dol scale provides a satisfactory basis for estimating pain intensity in the following respects:

a. It provides a numerical scale of sensory steps all of which are equal, even though the stimuli differences corresponding to these steps are not equal.

b. It is based on a type of stimulus which gives reproducible results in terms of threshold measurements.

c. It affords a basis for the intercomparison of other methods of estimating pain intensity, providing these estimates have been made in terms of a reproducible pain.

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FOREWORD

The investigations described in the papers comprising this malaria supplement were suggested and supported by the Office of Scientific Research and Development under the guidance of the Board for the Coordination of Malarial Studies. The work was done in cooperation with the Office of the Surgeon General of the U. S. Army, New York University, and the University of Chicago. Footnotes to each paper include references to others who assisted in one way or another.

For a number of reasons, the printing of these papers constituted an editorial and financial problem which threatened to prevent publication. Realizing the importance of making the results of these investigations generally available, the Malaria Study Section of the National Institute of Health volunteered to cooperate with the editorial staff of the Journal of Clinical Investigation in preparing the manuscripts for a special malaria supplement. To meet the financial difficulty, the following firms generously agreed to contribute toward the cost of publication and thus the supplement became possible: Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, Inc., and Wyeth, Inc.

The Editor-in-Chief of the Journal of Clinical Investigation delegated to the Malaria Study Section the editorial responsibility for critical evaluation of the material presented in this supplement. The manuscripts were read by appropriate members of the Section who acted as referees in regard to the experimental data and their presentation. The Section members feel that these papers constitute a notable contribution to our knowledge of antimalarial drugs and they are glad to have had an opportunity to assist in their publication.

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PROCEDURES USED AT STATEVILLE PENITENTIARY FOR THE TESTING OF POTENTIAL ANTIMALARIAL AGENTS¹

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(Received for publication December 23, 1946)

INTRODUCTION

The need for normal human subjects to use in appraising the activity of antimalarial drugs led, in 1944, to the establishment of a clinical research unit at the Illinois State Penitentiary, Stateville, Illinois. Through an arrangement with the Department of Public Safety,³ one floor of the prison hospital and a portion of a second floor were placed at the disposal of the Malaria Research Project. Approximately 500 inmates volunteered to act as subjects.

The studies were designed primarily to yield in-

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The studies were planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for the Coordination of Malarial Studies. This work was further aided by the participation of Army Medical officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, Inc., and Wyeth, Inc., for contributing toward the publication costs.

² Formerly Captain, M.C., A.U.S.

³ The authors wish to acknowledge the cooperation of the following officials in the State of Illinois who made these arrangements possible: Dwight H. Green, Governor, T. P. Sullivan, Director of Public Safety, and Joseph E. Ragen, Warden of Illinois State Penitentiary at Joliet-Stateville.

formation concerning the effect of potential antimalarial agents upon the relapse rate of sporozoite-induced *vivax* malaria. The observations also furnished information on the prophylactic and suppressive effects of the drugs tested, as well as data on their toxicology and pharmacology in man. The Chesson strain of malaria was selected for study because its short latent period between attacks (1) made feasible the rapid accumulation of information. An abundance of normal volunteers, in the younger age groups, living under standard conditions of diet and daily routine, made controlled clinical testing of antimalarial drugs possible. The institution being in a non-endemic area, accidental reinoculation was not a problem.

PROPHYLACTIC TESTS

The subjects were white, male inmate volunteers in good physical health. They were acquainted beforehand with the nature of the disease and the general plan of the study. Nearly all men were in the age group of 21 to 40. Only those whose stay in the institution was ascertained to be 18 months or longer were selected. Follow-up observations could be made in nearly 100 per cent of the subjects. Volunteers who had lived in known malarious areas, or who gave a history suggestive of previous malarial infection, or who belonged to one of the colored races, were excluded in order to minimize the factors of acquired or natural immunity.

A medical history was taken and physical examinations were made on all candidates. In addition, the following procedures were routine: complete blood count, urinalysis, urinary urobilinogen concentration, phenolsulfonephthalein excretion, cephalin-cholesterol flocculation, serum bilirubin, blood nonprotein nitrogen, blood Kahn, blood typing, chest x-ray, electrocardiogram, and, where indicated, erythrocyte sedimentation rate.

Malaria parasite. The Chesson strain of *P. vivax* (2) was isolated from a military patient who presumably had acquired his infection in New Guinea and was under treatment at the Harmon General Hospital, Longview, Texas (3). This strain is characterized by a high relapse rate when treated with noncurative drugs such as quinine and quinacrine, by a short period of latency between successive attacks, and by almost complete absence of delayed primary attacks (1, 4). The strain was maintained in psychotic patients at the Manteno State Hospital, Manteno, Illinois,⁴ chiefly by blood inoculations from donors who had manifested high gametocyte densities during trophozoite-induced infections. Not infrequently, however, donors were Stateville volunteers, whose malaria had been sporozoite-induced.

Transmission. Mosquito infection was carried out under the supervision of Drs. Clay G. Huff and Frederick Coulston of the University of Chicago, Department of Bacteriology and Parasitology. *Anopheles quadrimaculatus* mosquitoes were permitted to feed on patients whose gametocyte densities were such as to insure a reasonably high incidence of infection in the biting mosquitoes. They were then incubated for suitable intervals in the mosquito insectory at the University of Chicago.

Inoculation of volunteers. The technique of initiating sporozoite-induced infections was a modification of that used by Coatney and his coworkers (5) at the Federal Penitentiary at Atlanta, Ga. Mosquito lots were used in which the incidence of infection was high, as determined by random sampling. Each patient received the bites of ten infected mosquitoes. After feeding, the mosquitoes were dissected and a microscopic examination made of the salivary glands to determine the degree of infection. Each mosquito was kept in an individual plastic cylindrical cage 4.5 cm. in height, the ends of which were covered by coarse mesh. In most instances, the mosquito was per-

mitted to bite two or three subjects consecutively, the first feedings being interrupted before blood was visible in the abdomen of the mosquito. The last feeding was permitted to proceed until the mosquito stopped spontaneously or became engorged with blood. When a mosquito refused further feeding after it had already bitten one or two patients in a group, a substituted mosquito was allowed to complete the inoculation of the given group of men. Similarly, when mosquitoes were found on dissection to be uninfected, substitute mosquitoes were fed on the same subject.

In the multiple-bite technique, which was the usual procedure for inoculation, each man received, as nearly as possible, the same number of first, second, and third bites. Men receiving no drug and, therefore, serving as control subjects, were inoculated at the same time as those receiving drug for prophylactic testing. The group bitten by the same mosquitoes included at least one control subject.

The technique of infecting the subjects on inoculation day varied, and assumed one of several basic patterns, as follows:

1. Most commonly the patients were inoculated in groups of three.
2. The men were occasionally inoculated in pairs, each pair receiving the bites of the same ten infected mosquitoes.
3. In exceptional instances each subject was bitten separately by ten infected mosquitoes.

Administration of drugs. Three to five volunteers were employed for prophylactic tests. In the standard prophylactic procedure, the drug was administered on the day before inoculation, the day of inoculation, and for the following six days. Drugs were administered in gelatin capsules at four-hour intervals to obtain fairly constant concentration in the body fluids.

It was necessary to take elaborate precautions in order to avoid errors in dosage. At the start of observations, the exact number of capsules required for each patient's entire course of treatment was placed in individual bottles. An individual treatment sheet was also prepared. This sheet contained the following information: the patient's name, the code number of the drug, the

⁴ The facilities of the Manteno State Hospital were made available for these studies through the cooperation of Mr. Rodney H. Brandon and General Cassius Poust, former and present Directors of Public Welfare; Drs. Conrad Sommer and George A. Wiltrakis, former and present Deputy Directors, Department of Public Welfare, and Drs. Edward Ross and Walter H. Baer, former and present Superintendents of Manteno State Hospital.

detailed schedule of drug administration for the entire course of therapy, and the number of capsules that should remain in the bottle at the end of each day. The bottle and complete treatment schedules were given to the nurses. The capsules remaining in each bottle were counted daily and checked with the original schedule. At the time of the daily tally, enough capsules for the ensuing 24-hour period were removed from the original bottle and each dose was placed in a single small vial, on the label of which had been noted the name of the patient and the hour for administration. When an individual dose was administered, the nurse signed the treatment schedule opposite the hour for which the dose had been scheduled.

Observations. Thick films of the peripheral blood were taken daily after the eighth day following inoculation. When fever appeared, the films were read, and, if positive, previous films were examined. The films were of capillary blood and were prepared and stained according to the method of Earle and Perez (6). Sufficient area of a film was inspected microscopically to insure counting of all the parasites contained in 0.1 c. mm. of whole blood. The count was then referred to 1.0 c. mm.

The oral temperature and pulse were recorded when the thick films were made. Subjects were admitted to the hospital when fever or symptoms made ambulatory status inadvisable.

During the periods of hospitalization, observations were made as follows:

1. Rectal temperature, pulse, and respirations every four hours. When the rectal temperature was above 101° F., these observations were made every 30 minutes.
2. Blood pressure every day.
3. Total daily fluid intake and urine volume.
4. Daily urinalysis.
5. Determination of plasma drug concentrations every day or on alternate days; more frequently if special information was desired, as in the case of rapidly excreted or rapidly degraded drugs.
6. Special tests necessary to detect and study toxicity.

CURATIVE TESTS

Subjects. The volunteers were selected and inoculated as described for the prophylactic studies. In a few cases inoculation was achieved by the injection, intravenously, subcutaneously, or into lymph nodes, of a suspension of infected mosquito salivary glands. Many subjects had served in tests in which prophylaxis had failed, or had belonged to the control group. Only patients having primary attacks, first and second relapses, were used as subjects in tests where it was desirable to minimize the effects of immunity. Successive clinical attacks in the same individual were used to study the therapeutic properties of different drugs. Later observations (1), however, indicated that attacks in patients whose preceding latent period was prolonged, presented a milder therapeutic challenge to a test drug. Consequently, in the later curative trials whenever the amount of drug available was limited, tests were restricted to subjects whose preceding prepatent and latent periods showed no unusual prolongation.

Administration of drugs. For most therapeutic tests, treatment was begun at noon of the day after a patient had satisfied both of the following criteria:

1. Malarial parasites had been demonstrated in thick films of the peripheral blood for two consecutive days. The films were made as described above for prophylactic tests.
2. The rectal temperature had been 102° F. or over.

The drugs were administered at four-hour intervals to obtain fairly constant concentrations in the body fluids, except that slowly excreted or slowly degraded drugs, such as SN-8617, quinacrine, and chloroquine, were given less frequently. The usual period of drug administration was 14 days. The technique of drug administration was carried out as described above for the prophylactic tests.

Observations. Interim observations when patients were neither receiving drugs nor undergoing a malarial attack were made as follows:

Thick films of the peripheral blood were taken every other day and alternate films were studied

for the presence of plasmodia. The films were studied as outlined above. Thus, the onset of peripheral parasitemia was localized within one day. Patients were admitted to the hospital either when they became febrile or when symptoms or heavy parasitemia made ambulatory status inadvisable. When parasitic latent periods exceeded six months, observations were made once a week.

During hospitalization for therapeutic studies, the same observations were made as are listed above under Prophylactic Tests.

The majority of drugs studied in therapeutic trials have been members of the 8-aminoquinoline group of compounds. For these drugs, the following additional tests were added to the above routine:

- a. Leucocyte count every day and differential every fourth day, oftener when indicated.
- b. Hemoglobin and methemoglobin determination every day, and on occasion, twice a day.
- c. Electrocardiogram after treatment.

COMMENT

Using the technique outlined, it has been possible to develop a standardized procedure for the controlled clinical testing of new antimalarial agents. The actions of previously known drugs such as quinine, quinacrine, and pamaquin, have been documented quantitatively, and used as

standards of comparison in the evaluation of new drugs. Thirty new compounds have been tested.⁵

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⁵ The authors wish to acknowledge the services of the civilian nursing and technical staff and the assistance of Civilian Public Service assignees. These investigations would not have been possible except for the enthusiastic cooperation of several hundred inmate volunteers at Stateville Penitentiary. The volunteers are too numerous to list individually.

A STUDY OF THE PROPHYLACTIC EFFECTIVENESS OF SEVERAL 8-AMINOQUINOLINES IN SPOROZOITE-INDUCED *VIVAX* MALARIA (CHESSON STRAIN)¹

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(Received for publication December 23, 1946)

In 1931 and 1933, James (1 to 3) reported the first work on the prophylactic activity of the standard antimalarial agents, quinine, quinacrine (atabrine), and pamaquin (plasmochin), in *Plasmodium vivax* malaria under controlled conditions. He demonstrated that neither quinine nor quinacrine, given as prophylactic agents in therapeutic doses, prevented malarial infection. Extensive experience in the field (4 to 6) has corroborated this finding.

James (3) also reported that when pamaquin was given in amounts approaching the maximum tolerated dose for eight days, starting the day before sporozoite inoculation, none of the subjects developed malaria. Smaller doses protected only a few of the subjects.

However, in 1944, Feldman, Packer, *et al.* (7),

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The studies were planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for the Coordination of Malarial Studies. This work was further aided by the participation of Army Medical officers assigned to the project by the Surgeon General.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

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² Formerly Captain, M.C., A.U.S.

repeating the pamaquin observations of James, reported that in the McCoy strain of *vivax* malaria the same or slightly higher doses of pamaquin did not protect all of the subjects.

Because of the implication of these and other observations, that pamaquin has an extraordinary effect on the *P. vivax* parasite, an intensive study of pamaquin and its analogues was undertaken. We have conducted studies to determine whether pamaquin and other 8-aminoquinolines would prevent infection with the Chesson (Southwest Pacific) strain of *vivax* malaria.

TABLE I

The chemical structure, toxicity in the monkey, and antimalarial activity in birds of pamaquin and three other 6-methoxy-8-aminoquinolines (18)

Drug	Aliphatic side chain in 8-position	Comparative monkey toxicity	Quinine coefficient	
			Antimalarial activity	
			Chick Gallina-ceum	Duck Lo-phurae
Pamaquin	4-diethylamino-1-methylbutyl-amino	1	10-40	80
SN-1,452	3-aminopropylamino	$\frac{1}{2}$	40	3
SN-11,191	6-diethylaminohexylamino	$\frac{1}{2}$	30	100
SN-13,276	5-isopropylaminoamylamino	$\frac{1}{2}$	100	150

The four compounds studied (pamaquin, SN-11,191, SN-1,452, and SN-13,276 which is pentaquine) are all 6-methoxy-8-aminoquinolines. They differ from each other only in the structure of the aliphatic side-chain substituent of the 8-amino group (Table I). These four compounds were selected for human testing because they had high antimalarial activity in avian infections and because their toxicity in the monkey was low and of the pamaquin type, which, in contradistinction to the plasmacid type of toxicity, is reversible (8).

METHODS AND PROCEDURES

Details of the methods used in the drug-testing procedures are presented elsewhere (9). White, male, inmate volunteers with no history of previous malaria served as test subjects at Stateville Penitentiary.³

In order not to overlook a prophylactically effective drug by exhibiting the agent in insufficient dosage, an approximation of the maximum amount tolerated in man was administered. This dosage was calculated on the basis of toxicity in the monkey or in man when known. The regime used by James (1) in which the drug was given the day before inoculation, the day of inoculation, and for six days thereafter, was followed. In the patients to whom SN-1,452 and 180 mgm. per day of pentaquine (SN-13,276) were administered, the schedule was shortened because of serious toxicity. Because of their rapid disappearance from the plasma, the drugs were given in divided doses at four-hour intervals. All drug dosages are in terms of base. For example: 200 mgm. of the pamaquin naphthoate represents 90 mgm. pamaquin base.

The test and control subjects were inoculated by the bites of ten mosquitoes heavily infected with the Chesson strain of *P. vivax*. Since the penitentiary is in a non-endemic area, accidental reinoculation was no problem. The Chesson strain is characterized by the early appearance of the primary attack after prophylactic administration of suppressive drugs and by prompt relapse after therapy with non-curative drugs (10 to 12).

Thick films were made from capillary blood and examined for parasites at frequent and regular intervals throughout the follow-up period. Those subjects who developed malaria were hospitalized and treated with whatever drug regime was currently being used for therapeutic testing.

Blood was drawn daily about one hour after the 8 a.m. dose. The plasma concentrations were determined by the method of Brodie, Udenfriend and Taggart (13) modified as follows: 20 ml. of heptane and 0.5 ml. of iso-butyl alcohol were placed in a 60-ml. glass stoppered bottle. Ten ml. of plasma and 10 ml. of 0.1 N NaOH were added and the mixture shaken for 10 minutes. The mixture was then transferred to a 50-ml. centrifuge tube and centrifuged for five minutes. The water phase was aspirated and 15 ml. of the heptane phase was transferred to a 40-ml. glass-stoppered pointed centrifuge tube containing 0.5 ml. of coupling reagent (diazotized sulphanilic acid). The mixture was then shaken for five minutes and centrifuged. The heptane layer was removed by aspiration. Not less than 0.3 ml. of the water layer was transferred to a special microcuvette and the transmission read in a spectrophotometer (Coleman) at a wave length of 480 millimicra.

Hemoglobin and methemoglobin values were determined by the method of Wendel (14).

RESULTS

Prophylactic tests

Pamaquin, SN-11,191, and SN-1,452 were tested simultaneously in the early part of the study. Pentaquine was studied later.

Two of the five subjects who received pamaquin prophylactically developed fever and parasitemia promptly, 18 and 19 days after sporozoite inoculation (Table II). One subject developed a delayed primary attack 106 days after infection, and two volunteers have shown no evidence of

TABLE II

Prophylactic effect of four 6-methoxy-8-aminoquinolines

Drug	Daily dose*	Days of drug admin.†	Number of subjects	Number protected	Follow-up protected cases	Parasitic prepatent period, cases unprotected	Mean plasma concentration
	mgm.				months	days	gamma/liter
Pamaquin	90	1-1-6	5	2	20	19	80 70 90 370 80
SN-1,452	240	1-1-3	3	2		21	
SN-11,191	90	1-1-6	3	2		16	120 110 100
Controls	None		4	0		12 to 14	
SN-13,276	120	1-1-6	5	4	11 11 11 9	22	60 50 50 50 40
Controls	None		12	0		12 to 19	
SN-13,276	180	1-1-6 1-1-3 1-1-2	1 2 2	1 2 2	9		40 90 60 40 80
Controls	None		10	0		12 to 17	

* Dose is in terms of the base weight.

† 1-1-6 means drug administration the day before, the day of, and for six days after inoculation by the bites of ten mosquitoes.

malaria during the 20 months which have elapsed since they were infected.⁴ Two subjects who were treated with SN-11,191 and two who were treated with SN-1,452 have had no malaria during 20 months' observation (Table II). One subject

³ The studies reported in this paper would not have been possible except for the enthusiastic cooperation of the inmate volunteers and administrative officials of Stateville Penitentiary.

⁴ One subject showed two positive thick blood smears 362 days and 369 days after inoculation. Numerous negative smears and two negative sub-inoculations of 200 ml. of blood failed to substantiate the parasitemia. No fever developed.

in each of the latter groups developed clinical malaria within three weeks after inoculation.

The four subjects who served as controls for the first three drugs developed clinical malaria 12 to 14 days after inoculation.

Pentaquine was tested for prophylactic activity in doses of 120 mgm. and 180 mgm. per day. One of the five volunteers who were treated with 120 mgm. developed fever and parasitemia 22 days after inoculation. The other four subjects have shown no evidence of malaria during the nine to 11 months which have elapsed since they were bitten by infected mosquitoes. The 12 control subjects who were inoculated on the same days developed clinical malaria in 12 to 19 days.

All of the five subjects who received 180 mgm. of pentaquine per day have been protected from malaria to date, nine months after inoculation, even though two of them received the drug for only two days, and two more for only three days, after the day of inoculation. The ten volunteers who served as controls for this experiment developed clinical malaria 12 to 17 days after inoculation.

It is obvious that pamaquin exhibited prophylactic activity against the Chesson strain of *vivax* malaria, but it was only a partial prophylactic agent under the conditions of these studies. The prophylactic action of pamaquin was shared by three related 8-aminoquinoline compounds.

One subject in the pamaquin test group consistently maintained a plasma drug concentration about four times that attained by the other men in his group. He was the first subject in the group to develop malaria. In the group tested with SN-11,191, the one subject who developed malaria was the man who had the highest mean plasma concentration of the drug.⁵ Otherwise there was no observable correlation between concentration of drug in the plasma and success of prophylaxis.

Subsequent course of malaria in patients who were not protected by prophylactic treatment

Of the 21 patients who were treated prophylactically with these four 8-aminoquinolines, only six developed malaria. The six patients served as

TABLE III

Results of treatment of the primary attack in the six patients whose primary attack occurred after prophylactic treatment

Treatment of primary attack

Case	Previous prophylactic drug	Drug regime during primary attack	Result after treatment of primary attack	Relapse rate of other patients on same therapeutic regime
1	Pamaquin	SN-11,437	No relapse in 18 months	4 out of 4
2	Pamaquin	SN-11,437 and chloroquine (SN-7618)	No relapse in 18 months	4 out of 4
3	Pamaquin	Pamaquin (15 mgm./day) and quinine	No relapse in 14 months	3 out of 4
4	SN-1,452	SN-11,437 and quinine	Prompt relapse	3 out of 4
5	SN-11,191	SN-11,191	No relapse in 19 months	4 out of 4
	4 Controls infected by same mosquitoes as above patients	Various suppressive drugs	Relapse	
6	SN-13,276	Quinine	Delayed relapse	14 out of 16
	5 Controls infected by same mosquitoes as above patients	Various suppressive drugs	Relapse	

subjects in therapeutic tests at the time of their primary attacks. Each one was treated on a different drug regime with an antimalarial agent ineffective in preventing relapse in other cases (Table III). The one subject who was not protected by SN-1,452 relapsed promptly after treatment of his primary attack, and the subsequent course of his malaria was not unusual. The four subjects who were not protected by pamaquin or SN-11,191 have shown no evidence of malaria for the 14 to 19 months which have elapsed since the treatment of their primary attacks with non-curative drug regimes. The one patient who was not protected by SN-13,276 given prophylactically, relapsed after quinine treatment of the primary attack but had a long latent period.

⁵ At the time these observations were performed, the method for determination of SN-1,452 was not available.

The increased susceptibility of the primary attacks to drugs, after prophylactic treatment with 8-aminoquinolines, is supported by results in two sets of controls. First, 32 out of 36 patients similarly treated for a clinical attack, but infected by different mosquitoes, relapsed. Second, all of the control subjects, who were inoculated by the same mosquitoes as were the groups who received prophylactic drugs, and who were later treated with known suppressive drugs, experienced the frequent and repeated relapses characteristic of Chesson infections. These two groups serve as controls, therefore, not only on the effectiveness of drugs but also on the degree of infectivity of the mosquitoes.

TOXICITY

Since in these studies drugs were administered in doses approaching the estimated maximum tolerated dose, toxicity was expected and found. The symptoms produced by the drugs, with a few exceptions, were qualitatively alike. Many of the patients suffered abdominal, usually epigastric, discomfort or pain, anorexia, nausea, and vomiting. The epigastric pain, which was most severe in several of the group on 180 mgm. of SN-13,276, extended into the retrosternal area, and caused enough tenderness to limit the respiratory excursion.

Cyanosis was noted when the methemoglobin exceeded 6 or 7 per cent of the total hemoglobin. The average total hemoglobin values fell slowly and gradually. The average loss of hemoglobin was 1.75 grams per 100 ml. of blood, the greatest loss being about the 12th to 14th day after the start of medication. The white counts were more erratic, both mild leucopenia and slight leucocytosis being observed.

Electrocardiograms showed in many cases a slight and transient diminution in the height of the T-waves.

Other laboratory observations, including urinalysis and tests for urine urobilinogen, cephalin flocculation, estimations of the non-protein nitrogen and the serum bilirubin, yielded normal values or negative results. Postural hypotension, which occurred in some cases receiving pentaquine therapeutically (15), was not noted in these subjects.

Drug fever appeared on the fourth to sixth day of treatment in all three subjects receiving SN-1,452 and in two of the five receiving 180 mgm. of pentaquine daily.

Because of drug fever or abdominal pain, the administration of the drugs was terminated in the three cases on SN-1,452 and in four of the five on 180 mgm. of pentaquine prior to the scheduled end of treatment.

Although in individual cases a correlation was not always apparent, generally speaking, in groups of patients the degree of methemoglobinemia furnished a numerical guide not only to the amount of cyanosis, but also to the severity of other symptoms. The average per cent of hemoglobin converted to methemoglobin on the three consecutive days when methemoglobinemia was greatest is shown in Table IV.

TABLE IV
Methemoglobin formation during administration of several 8-aminoquinolines

Drug	Daily dose (base)	Methemoglobinemia
	mgm.	Per cent of total hemoglobin
Pamaquin	90	11.7
SN-1,452	240	10.3
SN-11,191	90	9.6
Pentaquine	120	5.3
Pentaquine	180	11.0

Inasmuch as most of the courses on 180 mgm. of pentaquine and all the courses of SN-1,452 were terminated early, the methemoglobin percentages for these groups are probably lower than would have been the case had they been allowed to continue for the full eight days. Peak values for methemoglobinemia were commonly reached after seven days. The regime of 120 mgm. of pentaquine per day caused less methemoglobin formation than any of the other regimes. The subjective symptomatology was also less in this group.

DISCUSSION

In these investigations, drug administration was limited to six days after inoculation because it has been shown by subinoculation that trophozoites usually do not appear in the peripheral blood before the ninth day after the mosquito bites (4). James' eight-day regime, with inoculation on the

second day, insures maximum concentration of the time of sporozoite inoculation and throughout that portion of the incubation period in which presumably only pre-erythrocytic forms of the malarial parasite are present. Because the 8-aminoquinolines disappear from the plasma within 24 hours after the last dose, and because extensive tissue accumulation does not occur (16), any effect produced may be assumed to be the result of action on pre-erythrocytic stages. The fact that pamaquin and three of its analogues exhibited prophylactic activity against *vivax* malaria when administration was limited to the prepatent period of the disease, suggests that these compounds possess the ability to inactivate or eradicate the sporozoites or hypothetical per-erythrocytic or tissue stages.

Four of the six subjects who developed primary attacks in spite of prophylactic treatment were then cured with drugs ordinarily ineffective in preventing relapse. In these subjects, the initial treatment with 8-aminoquinoline compounds may have altered the parasites sufficiently to render them unusually susceptible to subsequent therapy. Since the previous prophylactic treatment was stopped before the time when trophozoites appear in the untreated case, any effect was presumably exerted only on pre-erythrocytic stages of the parasite.

It is our opinion that 180 mgm. of pentaquine for eight days exceeds the maximum safe dose in man. Drug fever precludes the administration of SN-1,452 at 240 mgm. a day. Pamaquin at 90 mgm. a day, SN-11,191 at 90 mgm. a day, and pentaquine at 120 mgm. a day also exceed the dosage tolerated by some white healthy subjects. Their clinical use in these amounts is dangerous.

The drugs studied have relatively high toxicity for man and are quickly degraded or metabolized so that they must be administered at frequent intervals. These qualities preclude the general use of these compounds as prophylactic agents, though one of the four (pentaquine) has been demonstrated to be highly effective in the radical cure of *vivax* malaria when administered with quinine in relatively non-toxic doses (17).

CONCLUSIONS

1. Using the Chesson strain, we have confirmed the finding of James, and of Feldman, Packer, *et*

al., that pamaquin may act as a true prophylactic for sporozoite-induced *Plasmodium vivax* malaria. However, it did not protect all subjects under the conditions of this study.

2. Three other 6-methoxy-8-aminoquinoline compounds were at least equally effective as prophylactic agents when administered in a dosage approaching the maximum amount tolerated.

3. There was no positive correlation between plasma drug concentration and the prophylactic effect of these drugs.

4. In those subjects whose primary attacks were not prevented by prophylactic treatment, there is evidence that the disease was so altered by the prophylactic therapy that it was rendered susceptible to cure by drug regimes ordinarily ineffective in preventing relapse.

5. The results suggest that the compounds tested exert a deleterious effect upon pre-erythrocytic stages of the malarial parasite.

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THE USE OF SN-10,275 IN THE PROPHYLAXIS AND TREATMENT OF SPOROZOITE-INDUCED *VIVAX* MALARIA (CHESSON STRAIN)¹

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A large number of piperidyl quinolinemethanols were investigated for antimalarial activity in avian infections because of close chemical similarity to quinine, as part of the wartime search for new and more effective antimalarial agents. In preliminary observations, SN-10,275,³ or 6,8-dichloro-2-phenyl- α -2-piperidyl-4-quinolinemethanol (Figure 1), gave greatest indication of promise. It is a compound of the Ainley-King series, which has a high degree of antimalarial activity in cathemerium and lophurae malaria in the duck and moderate activity in gallinaceum and lophurae malaria in the chick (1).

PROCEDURES AND METHODS

General. Details of the general procedure and the plan of observations are reported elsewhere (2). Healthy

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The studies were planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for the Coordination of Malarial Studies. This work was further aided by the participation of Army Medical officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

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² Formerly Captain, M.C., A.U.S.

³ Developed by Buchman and Koepfli and collaborators at the California Institute of Technology.

Caucasian volunteers⁴ at Stateville Penitentiary were infected with Southwest Pacific vivax malaria (Chesson strain) (3) by the bites of infected *Anopheles quadrimaculatus* mosquitoes. This strain is characterized by a high relapse rate when treated with non-curative drugs such as quinine and quinacrine (atabrine), by a short period of latency between successive attacks, and by almost complete absence of delayed primary attacks. In the tests for prophylaxis, three test subjects and three controls were inoculated by bites from the same group of mosquitoes. In the tests for therapeutic activity, five similarly inoculated volunteers undergoing primary attacks or second relapses were treated soon after the appearance of parasitemia.

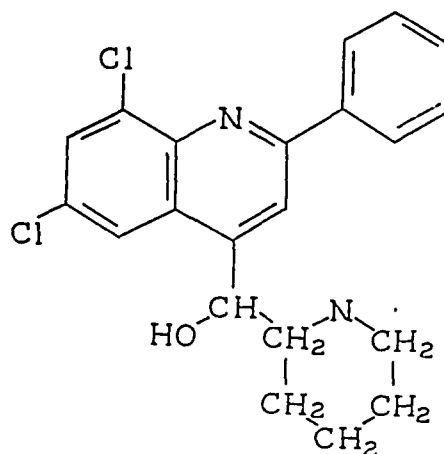


FIG. 1. STRUCTURAL FORMULA OF SN-10,275

Selection of patients. When a series of attacks of (Chesson) malaria, as observed under these standardized conditions, was divided (4) into two groups according to the length of the prepatent and preceding latent periods, there was a significant difference in relapse rate between the groups after treatment with suppressive drugs. The group with short intervals showed a relapse rate of 98 per cent, whereas the group with the longer intervals had a rate of 67 per cent.

In the therapeutic tests, SN-10,275 was administered to two individuals whose attacks belonged to the first group and, therefore, constituted a severe therapeutic

⁴ The observations reported in this paper would not have been possible except for the enthusiastic cooperation of the inmates and administrative officers at Stateville Penitentiary.

challenge. It was also tested against three attacks which fell into the group offering a milder challenge.

Drug administration. One gram of SN-10,275 monohydrochloride (equivalent to 0.859 gram of base) per day was administered in four to six divided doses. The subjects in the prophylactic test received drug on the day before, the day of, and for six days after inoculation. The therapeutic trial was conducted at the same daily dosage but consisted of a 14-day course of treatment.

Determination of drug in the plasma. Whole oxalated blood was centrifuged for 15 minutes at 2,000 r.p.m.; the plasma was separated and re-centrifuged for 60 minutes at the same speed to insure complete removal of the components of the buffy coat.

SN-10,275 was analyzed by the method of Butler (5) modified as follows: Five ml. of 0.2 M Na_2HPO_4 and 1 ml. of non-fluorescent absolute alcohol were placed in a 50-ml. glass stoppered centrifuge tube. Two or 3 ml. of plasma were added to the mixture followed by 15 ml. of purified non-fluorescent benzene. The mixture was shaken for 15 minutes, centrifuged and the water layer aspirated. The benzene phase was decanted into 8 ml. of 10 per cent NaOH and shaken for five minutes. After centrifugation and aspiration of the alkali layer, exactly 10 ml. of the benzene layer were transferred to a 50-ml. glass stoppered centrifuge tube containing 10 ml. of 10 N H_2SO_4 . After the mixture was shaken for 15 minutes, centrifuged, and the benzene layer aspirated, the acid layer was transferred

to cuvettes. The fluorescence was read in a Coleman 12 A photofluorometer with B-1-S and PC-1 filters.

Recoveries and standards were run simultaneously with the unknown.

RESULTS

Prophylactic effect. The results are summarized in Table I. All three patients developed parasitemia and fever. However, the prepatent periods in the test group were six to nine times as long as those in the control group. The plasma concentrations at approximately the time of appearance of parasitemia ranged from 66 gamma per liter to 110 gamma per liter.

Therapeutic effect. The results are summarized in Table II. The two cases that presented a severe therapeutic challenge relapsed. Of the three cases that offered only a moderate challenge, one relapsed. The others have been followed for over a year. Drug disappeared from their blood plasma 108 and 135 days after end of therapy.

Following treatment with SN-10,275, the latent periods of those individuals who relapsed were 296, 107 and 99 days. The median latent period observed with quinine and quinacrine in the Chesson strain of malaria under the conditions of this investigation, is 15 and 34 days, respectively (6). The plasma concentration of SN-10,275 at approximately the time of appearance of parasitemia ranged from 59 to 80 gamma per liter.

Concentration of SN-10,275 in the plasma. SN-10,275 remained in the plasma for long periods after medication had been discontinued. The falling curves of plasma concentration are shown for eight subjects in Figure 2. There was a wide variation in rate of fall from individual to individual. One subject differed markedly from the remainder of the group in that the drug persisted

TABLE I

Prophylactic effect of SN-10,275 on sporozoite-induced P. vivax malaria (Chesson strain)

Patient	Dose (base)		Mean plasma concentration during therapy	Prepatent period (parasitemia)	Plasma concentration at time of clinical attack
	Daily	Total			
	grams	grams	gamma per liter	days	gamma per liter
1	0.859	7.0	680	66	66
2	0.859	7.0	860	97	81
3	0.859	7.0	530	74	110
Three controls				12,13,12	

TABLE II

Therapeutic effect of SN-10,275 on vivax malaria (Chesson strain)

Patient	Dose (base)		Mean plasma concentration during therapy	Type of therapeutic challenge	Latent period (parasitemia)	Plasma concentration at time of relapse	Length of observation since end of therapy in negative cases
	Daily	Total					
	grams	grams	gamma per liter		days	gamma per liter	days
1	0.859	12.0	1,200	Moderate	—	—	421
2	0.859	12.0	550	Moderate	—	—	421
3	0.859	12.0	1,500	Moderate	296	64	—
4	0.859	12.0	1,400	Severe	99	80	—
5	0.859	12.0	1,200	Severe	107	59	—



FIG. 2. CONCENTRATION OF SN-10,275 IN THE PLASMA OF EIGHT INDIVIDUALS AFTER ORAL ADMINISTRATION WAS DISCONTINUED

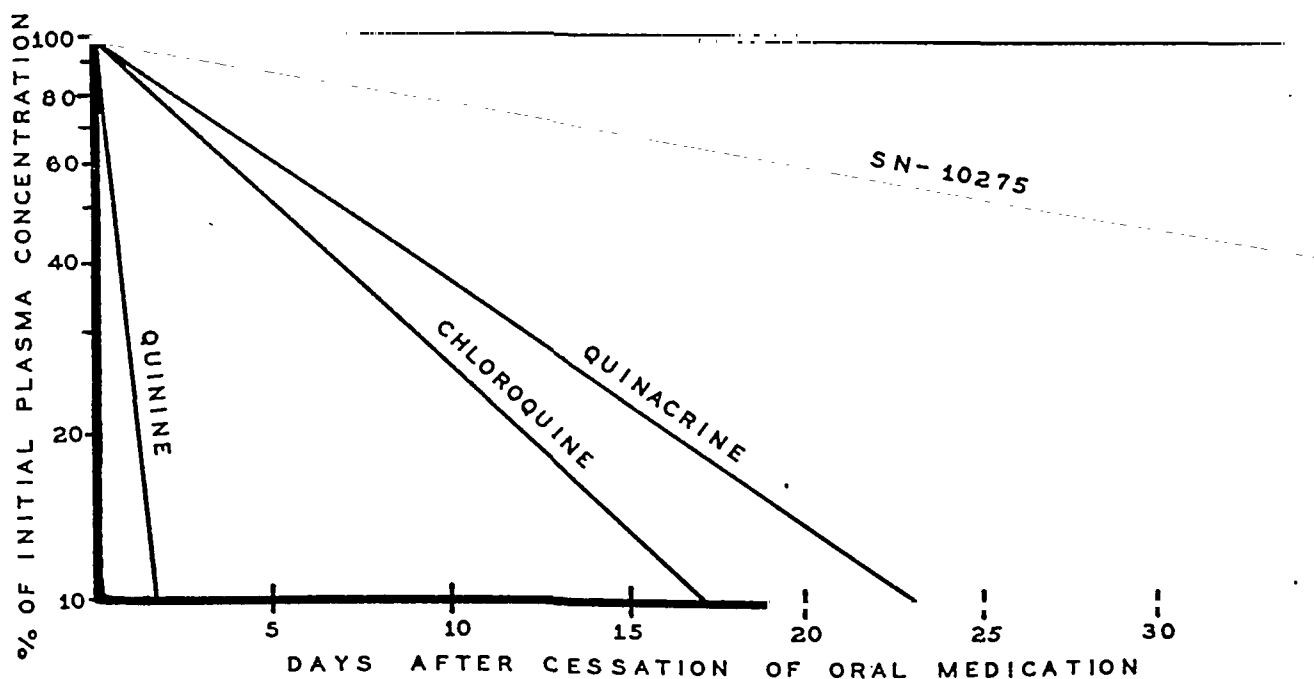


FIG. 3. RATE OF DISAPPEARANCE FROM THE PLASMA OF QUININE, CHLOROQUINE, QUINACRINE (ATABRINE), AND SN-10,275

Schematic representation in terms of per cent of initial plasma concentration. Curve for SN-10,275 plotted on basis of loss of 20 per cent per week as derived from curves presented in Figure 1. Curves for quinacrine and chloroquine plotted on basis of losses of 50 per cent per week (7) and 60 per cent per week (8), respectively.

in his plasma more than twice the average time. The concentration of the drug in plasma decreased with time in approximately exponential fashion, the mean loss for the majority of the group being approximately 20 per cent per week. This is a much lower rate of disappearance than that shown by quinine and quinacrine (atabrine). Figure 3 illustrates schematically the comparative rates of decrease of plasma concentration of quinine, quinacrine, SN-10,275 and also that of the recently developed antimalarial, chloroquine (6, 8).

Toxicity. Eight patients received SN-10,275 at a dosage of 1.0 gram of the salt per day. One patient experienced mild gastrointestinal symptoms consisting of cramps, nausea and mild diarrhea during treatment. Another individual had fever of 102° F., headache, and backache towards the end of the 14-day course of therapy. Physical examination and laboratory studies gave negative results or normal values. The fever persisted for about 12 hours. Three doses of the drug were omitted, but since the entire episode appeared innocuous, therapy was resumed with no untoward effects.

All eight patients manifested photosensitivity of the skin. This varied from a slight tingling of the facial skin to severe burning sensations in the same area accompanied by erythema. One individual had some desquamation of the skin of the nose, and another had mild labial edema. These symptoms appeared only after exposure to sunlight for periods of 15 minutes or more. With the exception of one individual who still had symptoms and detectable concentration of drug in the

plasma ten months after he received SN-10,275, this sensitivity to sunlight persisted for one-half to four months, gradually diminishing in intensity. Two subjects manifested increased irritability of the skin when subjected to mild mechanical trauma such as shaving or rubbing. Another patient noted smarting and burning of the eyes after exposure to sunlight. As shown in Table III, the severity and duration of these symptoms are roughly correlated with the mean concentration of SN-10,275 in the plasma during therapy. Abnormal amounts of porphyrins could not be detected in the urine of three patients.

SUMMARY AND CONCLUSION

In the prophylactic tests, SN-10,275 did not prevent the development of malaria. Parasitemia appeared when the plasma concentrations had fallen to 66–110 gamma per liter. This required 66 to 97 days, a period six to nine times as long as the usual prepatent interval in controls.

In the therapeutic tests against attacks presenting a mild therapeutic challenge, one out of three patients underwent further relapse. This occurred when the plasma concentration had fallen to 64 gamma per liter, 296 days after termination of treatment with SN-10,275. In the tests against the two attacks offering a severe therapeutic challenge, both patients suffered further relapse. Parasitemia appeared when the plasma drug levels fell to 59 and 80 gamma per liter, requiring 99 and 107 days, respectively.

The observed prolongation of the prepatent and latent periods may be attributed to the persistence

TABLE III
Clinical toxicity of SN-10,275 in eight volunteers

Patient	Mean plasma concentration	Facial tingling	Facial erythema	Facial edema	Duration	Comment
	<i>gamma per liter</i>				<i>months</i>	
1	1,500	+++	+++	+	10	Hyperirritability of skin on mechanical trauma. Slight hyperirritability of skin on mechanical trauma. Mild photophobia. Desquamation of nasal skin following sun burn on one occasion. Headache, backache, and fever during drug administration.
2	1,400	+++	0	0	4	
3	1,200	+++	+++	0	2	
4	1,200	++	+	0	$\frac{1}{2}$	
5	860	++	+	0	1 $\frac{1}{2}$	Fair complexion.
6	680	++	0	0	$\frac{1}{2}$	
7	550	+	0	0	$\frac{1}{2}$	
8	530	+++	+++	0	1 $\frac{1}{2}$	

in the body fluids of SN-10,275 for long periods of time. Butler (9) has shown that the drug is almost entirely in undegraded form.⁵ The concentration of drug in the plasma at the time that parasitemia became patent was of the same order of magnitude in all patients studied. One individual showed a latent period after treatment three times that of the others. However, in this individual the rate of loss of drug was much lower, so that at the time of relapse, his plasma concentration was within the range observed in the other patients.

Similarly, the same individual showed persistence of photosensitivity far longer than the remainder of the group. The degree of tingling of the skin showed a positive correlation with the initial plasma concentration achieved, and the duration of this symptom was roughly proportional to the rate of loss of drug from the plasma.

The toxic manifestations and the variation in rate of disappearance from the body of SN-10,275 limit the value of the drug as a suppressive agent. However, further investigation of constitutionally related compounds is indicated because a non-toxic drug which retained the antimalarial activity of SN-10,275 and remained in the body for long periods of time, would have great value in the chronic suppression of malaria.

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⁵ It is of interest to note that quinine (10 to 12) and other cinchona alkaloids (13, 14) are degraded in the body by oxidation on the 2-position of the quinoline ring to form carbostyryls. SN-10,275 has a phenyl group on the 2-position and this substitution may account for the apparent absence of degradation products in the plasma.

THE TOXICITY OF LARGE DOSES OF PENTAQUINE (SN-13,276), A NEW ANTIMALARIAL DRUG¹

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This paper reports observations on the toxicity of pentaquine³ when administered in amounts higher than the recommended therapeutic dose (1). The purpose of the observations was to define the margin of safety of the drug in clinical use.

Pentaquine (SN-13,276) offers considerable promise as a radical cure of *vivax* malaria (1, 2). It is closely related chemically to pamaquin (Figure 1). At dosage levels *within* the therapeutic range, the drug has seldom caused serious toxic reactions in white subjects. We have reported therapeutic results in 88 patients treated with 60 mgm. of base per day for 14 days (2). Most of them had few or no symptoms; only two subjects had severe symptoms, but not such as to make discontinuance of medication advisable. Symptoms, when they occurred, were similar to those produced by pamaquin: methemoglobinemia, abdomi-

nal discomfort or pain, anorexia, nausea, and vomiting. No hemolytic crisis occurred, but none of the subjects were negroes, in whom the incidence of hemolytic episodes is high with pamaquin (1). Sixty mgm. of pentaquine base have a toxicity approximately equivalent to 30 mgm. of pamaquin base.

Pentaquine was administered in double or triple the maximal therapeutic dose of 60 mgm. per day in order to explore the prophylactic (3) and curative effect of the drug in *vivax* malaria and to define the upper dosage limits tolerated in man.

PROCEDURE

Details of the routine procedures used in these studies are reported elsewhere (4). The subjects were healthy, white, inmate volunteers⁴ in the Illinois State Penitentiary at Stateville. In prophylactic tests, the drug was administered to ten subjects at four-hour intervals for eight days at a daily dose of 120 or 180 mgm. of base. Inoculation with the bites of mosquitoes was performed

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, Inc., and Wyeth, Inc., for contributing toward the publication costs.

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³ Pentaquine was synthesized by Dr. Nathan Drake at the University of Maryland.

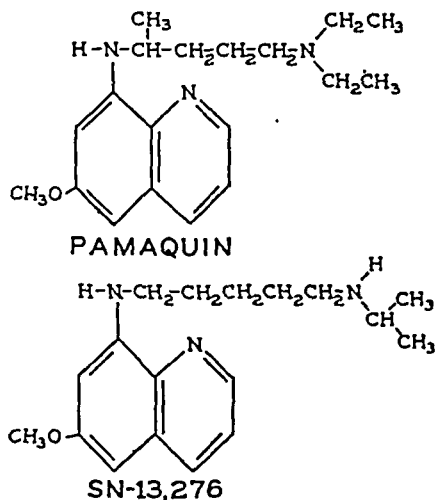


FIG. 1. STRUCTURAL FORMULAE OF PENTAQUINE (SN-13,276) AND PAMAQUIN (PLASMOCHIN)

⁴ These studies would not have been possible except for the enthusiastic cooperation of the inmate volunteers and administrative officials of Stateville Penitentiary.

on the second day of drug administration. In high-dosage therapeutic tests, 120 mgm. of pentaquine base was administered daily for 14 days to ten subjects who had acute clinical attacks of malaria at the start of treatment. In half of this latter group, 2.0 grams of quinine sulfate was administered daily concurrently with pentaquine. Certain individuals in both the prophylactic and high-dosage therapeutic trials failed to complete the scheduled course of treatment because of toxic reactions.

The volunteers were hospitalized during the entire course of drug administration and were interviewed for symptoms at least once daily. The following observations were made in all cases: rectal temperature, pulse and respiration every four hours; fluid intake and urinary output, blood pressure, methemoglobin and hemoglobin determinations, and urinalysis daily; leucocyte count every two days; and differential count every four days. An electrocardiogram was made after treatment; and in most instances, non-protein nitrogen and serum bilirubin determinations, cephalin-cholesterol flocculation tests, and urinary urobilinogen estimations were performed at the end of the treatment period.

Hemoglobin and methemoglobin were estimated by the photocolometric method of Wendel (5). The concentration of drug in the plasma was determined by the modified (3) method of Brodie, Udenfriend and Taggart (6). Oxygen content and capacity and carbon dioxide content of the arterial and venous blood were determined by the gasometric method of Van Slyke and Neill (7) using arm blood.

The venous tone was measured in three patients by the use of a plethysmograph. The dilatation of the loosely gloved hand, when the venous pressure was increased from 10 to 50 mm. Hg by applying pressure to the forearm through a sphygmomanometric cuff, was measured by the displacement of water. An interval of normal pressure was allowed after every reading, and the water was kept at 37° C. in order to avoid errors due to edema and reactive hyperemia. The average of at least five determinations was used. The results were expressed in cubic centimeters per liter of hand volume in a manner similar to that used by Wilkins, Haynes and Weiss (8). Increased volume of the hand over that of normal controls was interpreted as implying decreased venous tone.

RESULTS

For convenience in analysis and presentation, the 20 subjects are divided into four groups of five volunteers as follows (Table I).

1. *Therapeutic test at 120 mgm. base per day with quinine for 14 days.* Drugs were administered concurrently in the treatment of clinical attacks.

2. *Therapeutic test at 120 mgm. base per day without quinine for 14 days.* The clinical attack was treated with pentaquine alone.

TABLE I

Case no.	Type of trial	Daily dosage	Duration of drug administration	Epigastric or sub-sternal pain†	Weakness, prostration†	Syncope, postural hypotension†	Hemoglobin		Weight loss or gain	Antimalarial result
							Converted to methemoglobin	Change total hemoglobin		
		mgm.	days				gm. per 100 cc.	gm. per 100 cc.	kgm.	
1	Therapeutic	120 with quinine	14	++++			1.3	+0.4	- 3	No relapse in 12 months
2			14	++++			1.0	-0.2	- 3	No relapse in 12 months
3			14	++			0.8	+0.3	- 4	No relapse in 12 months
4			6½*	++++	+++		0.7	+0.2	0	No relapse in 12 months
5			14	++++			0.6	-0.2	- 2	No relapse in 12 months
6		120	9½	++++			1.3	0	- 5	No relapse in 12 months
7			14	++	++++	++++	2.0	-3.4	-11	Relapsed in 14 days
8			14	++			1.8	-2.1	- 3	Relapsed in 26 days
9			14	++++	++++	++++	1.5	-3.9	-10	No relapse in 12 months
10			14	++	+++	++	2.1	-3.6	0	No relapse in 12 months
11	Prophylactic	120	8	+			0.5	-0.8	+ 1	No attack in 13 months
12			8	++			1.3	-1.2	+ 2	No attack in 13 months
13			8	+++			0.8	-2.3	+ 2	No attack in 13 months
14			8	+++			0.9	-1.9	0	Primary attack 22 days
15			8				0.9	-1.4	+ 4	No attack in 13 months
16		180	5½*	++++	++		2.2	-1.9	0	No attack in 12 months
17			4½*	++++			1.2	-1.8	0	No attack in 12 months
18			8	+++			1.1	-1.1	+ 3	No attack in 12 months
19			4½*	++++	++		2.7	-1.4	+ 2	No attack in 12 months
20			5½	++++	++		1.9	-1.4	- 1	No attack in 12 months

* Drug fever.

† + to ++++ represents severity of symptom.

3. *Prophylactic test at 120 mgm. base per day for eight days.* These subjects did not have clinical malaria and did not receive quinine.

4. *Prophylactic test at 180 mgm. base per day for eight days.* These subjects were treated as in the previous prophylactic test but with a higher dosage of the drug.

The most common symptom was pain which was present in 19 of the 20 volunteers, and was severe in 13. Usually, after the first two or three days, the patients noted an epigastric discomfort, which in many became greatly aggravated and seemed to spread into the precordium or the retrosternal area. In a few patients the pain radiated to one or the other shoulder, to the back or to the neck. The ache or pain was constant with periodic exacerbation, and was accompanied in most cases by epigastric tenderness. In several individuals the epigastric tenderness was severe enough to limit the respiratory excursion, resulting in shallow, rapid respiration and "shortness of breath." A few patients described exacerbations of the pain after taking doses of the drug. In none was it apparently related to exercise, meals, defecation, or micturition. Efforts to relieve the pain with adrenalin or atropine were unsuccessful. The ordinary analgesics were withheld in order not to mask the signs of pentaquine toxicity.

In many instances the pain diminished or disappeared after the first three to eight days of treatment, but in others it persisted throughout treatment, subsiding gradually within the next two or three days. In one instance (120 mgm. daily therapeutically with quinine) unexplained but very mild chest discomfort persists six months later.

Other transient symptoms occurring with less uniformity in the various groups, included anorexia, nausea, vomiting, headache, weakness and prostration.

Except for cyanosis, which was present whenever methemoglobinemia exceeded 6 or 7 per cent and frequently was detectable at lower values, few abnormalities were found on physical examination. Slight pallor was sometimes seen. The occurrence of drug fever on the fifth to seventh day of treatment necessitated discontinuation of the drug in three subjects. In a fourth, slight fever oc-

TABLE II
The toxicity of pentaquine in doses greater than the therapeutic dose

Pentaquine regime (daily dose)	Methemoglobin formation (average)	Approximate pamaquin equivalent (daily dose)
mgm.	per cent of total hemoglobin	mgm.
120 prophylactic	5.4	45
180 prophylactic	13.1	90*
120 with quinine therapeutic	5.6	60
120 without quinine therapeutic	11.3	90*

* Although the amounts of methemoglobin formed were almost the same, the second and fourth pentaquine regimes produced toxicity considerably in excess of that caused by 90 mgm. of pamaquin daily.

curred on the sixth day, treatment having been discontinued on the fifth day for other reasons.

A gradual loss in total hemoglobin commonly occurred, but no hemolytic crisis developed. All the subjects had methemoglobinemia. Using pamaquin as a standard of comparison, on the basis of the average amount of methemoglobin formed, the four groups may be compared with each other and with pamaquin (Table II). Very high degrees of methemoglobinemia were observed in the group at 180 mgm. alone and in the therapeutic group at 120 mgm. without quinine. In the latter group, a loss of total hemoglobin combined with conversion of hemoglobin to methemoglobin produced a marked reduction in the blood pigment available for oxygen transport.

There was a diminution in the height of the T-waves in the electrocardiograms in most of the subjects. The T-wave amplitude was reduced in some or all leads. Inversion of T-waves in Leads I and IV F occurred in one case. Occasionally also, an initially inverted T_s became shallow or upright. The electrocardiographic changes were reversible.

In addition to the symptoms, signs and laboratory observations described above which were common to all the groups, serious physiological abnormalities were present in three of the subjects on 120 mgm. daily without quinine, given therapeutically. These patients displayed postural hypotension with syncope, arterial and venous oxygen unsaturation, and weight loss, which persisted for a long time after the end of treatment.

The three subjects were healthy, white males

29 to 36 years old. They were inoculated, along with other volunteers, on March 2, 1946, with *Plasmodium vivax* (Chesson strain) by the bites of ten infected mosquitoes. Parasitemia and fever followed in 12 to 16 days, and the drug was begun within two days after the onset of fever. Pentaquine was given at a daily dose of 120 mgm. of the base, 20 mgm. being administered every four hours day and night for 14 days. Mean concentrations of the drug in plasma ranged between 48 and 103 gamma per liter. These concentrations did not differ significantly from those obtained in the volunteers less severely affected. The three subjects were afebrile, and the peripheral blood was free of parasites within four days of the end of treatment. The case records follow.

Case 9

During the first three days of treatment this patient complained of severe epigastric pain and tenderness, with periodic paroxysmal accentuation. During some of these extremely painful episodes an irregularity of the pulse was observed, which proved to be sinus arrhythmia by electrocardiograph. The pain disappeared spontaneously after three days, but profound weakness, nausea, and almost complete anorexia persisted throughout the two-week trial. On the sixth day of treatment the patient experienced an attack of syncope and subsequently fainted repeatedly on assuming the upright posture. Cyanosis was noted early and persisted until a week or more after the end of treatment.

Calculated on the basis of five-day averages, a fall of 3.9 grams in total hemoglobin occurred during treatment. This fall was gradual, there being no acute hemolytic episode. The erythrocyte count decreased proportionately. Methemoglobinemia developed rapidly, the maximal levels being achieved on the third day and a fairly constant level being maintained thereafter. The average methemoglobinemia for the last five days of treatment was 1.8 grams per 100 cc., or 13.6 per cent of the total hemoglobin.

Urinalysis and total and differential leucocyte counts were normal during treatment, and after treatment no abnormalities were found in the blood non-protein nitrogen, blood sugar, serum bilirubin, sodium, potassium, and protein, arterial and venous carbon dioxide content, the venous pressure, arm-to-tongue circulation time, blood and plasma volume (Evans' blue method), and roentgenogram of the chest. The electrocardiogram showed a diminution in the height of the T waves in Leads I, II, and IV F and a diminution of the previously inverted T in Lead III. The Wintrobe red blood cell indices were within normal limits.

In addition to the low oxygen-carrying capacity due to methemoglobinemia and anemia, there was a striking diminution in the arterial and venous oxygen content

TABLE III

Case	Time after beginning of 2-week treatment	O ₂ capacity	Arterial		O ₂ lost by blood in perfusing the tissues	Venous	
			O ₂ content	Oxygenation of hemoglobin		O ₂ content	Oxygenation of hemoglobin
no.	days	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent	per cent
9	13	16.45	13.98	85.3	10.94	3.04	18.5
	16	16.22	14.86	91.9	6.20	8.66	53.4
	28	18.16	16.54	90.7	4.11	12.43	68.2
7	38	19.40	17.40	89.6	9.06	8.34	43.0
10	13	14.75	12.68	85.7	8.17	4.51	30.2
	30	18.70	17.27	92.4	6.67	10.60	56.7
Normal blood		20.00	19.00	95.0	4.20	14.80	74.0

and in the oxygen saturation of the remaining hemoglobin (Table III). Two weeks after the end of treatment, abnormal oxygen unsaturation was still present, although less pronounced than before.

Syncope not only on standing but even in the sitting position was a prominent symptom. The recumbent blood pressure at the start of treatment was 128 mm. Hg systolic and 88 diastolic. At the end of treatment it was 98 systolic and 64 diastolic. Standing tests demonstrated that, when the patient assumed the upright position, the pulse rate increased (as is normal), but the blood pressure not only failed to show the normal physiologic rise, but precipitously fell, pressures of 45 systolic and 30 diastolic being recorded within one minute after standing. In seven tests, performed three weeks after the end of treatment, the patient fainted in 42, 55, 68, 91, 46, 46, and 43 seconds. Even when he "marked time" in the erect position the subject fainted in 55 seconds. After 0.4 cc. of 1:1000 epinephrine was injected subcutaneously, syncope occurred after 16 seconds of standing.

Two months after the end of treatment the tests were repeated, using a table tilted to 55 degrees. The use of the inclined table resulted in delaying the occurrence of syncope, permitting more time for observations. Even after this two-month interval, a fall in the systolic and diastolic blood pressure and an increase in the pulse rate occurred (Figure 2) followed by syncope. The reaction of this patient (Figure 2) may be compared with that of a normal individual (Figure 3). Four months after treatment the patient tolerated a short period of motionless standing and had a preliminary rise in blood pressure before the fall.

On the theory that the unusual degree of oxygen unsaturation might be due to stagnation in a dilated venous bed, the venous tone was measured. The distensibility of the hand veins was found to be greater than that of ten control subjects (Table IV). This observation made

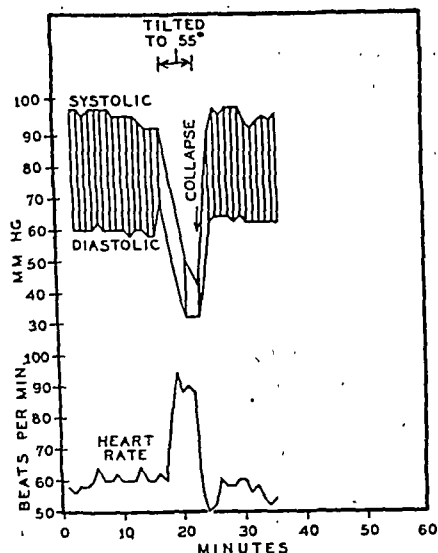


FIG. 2. CASE 9. THE FALL IN SYSTOLIC AND DIASTOLIC BLOOD PRESSURE AND THE RISE IN THE PULSE RATE UPON TILTING TO 55°, TWO MONTHS AFTER THE END OF PENTAQUINE THERAPY

two months after the end of treatment, was confirmed at three months and again at four months.

Profound anorexia persisted for a long time, resulting in a weight loss of 10 kgm. in six weeks which was slowly and incompletely regained, in contrast to the rapid recovery of lesser weight loss in other subjects.

The patient was discharged from the hospital two and a half months after the therapeutic course. Although he returned to his routine work in the prison tailor shop,

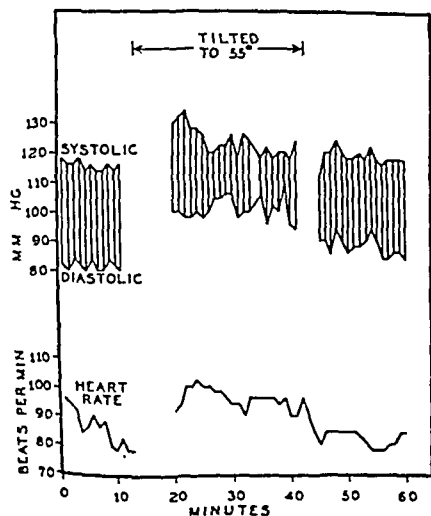


FIG. 3. CONTROL SUBJECT. THE RISE IN SYSTOLIC AND DIASTOLIC PRESSURES AND PULSE RATE UPON TILTING TO 55°

TABLE IV
Studies of hand volume

Case	Time after end of treatment	Increase in hand volume when venous pressure was increased from 10 to 50 mm. Hg
9	2 months	<i>cc. per liter</i> 24
	3 months	20
7	2 months	24
	3 months	22

10 controls		Range	Mean
		8-20	15

he still has (four months after the test) giddiness in the morning, increased fatigability, and absence of erections and ejaculations.

Case 7

This volunteer suffered abdominal discomfort, similar to but milder than that of Case 9, lasting eight days; and he had more severe anorexia, nausea, vomiting, and weakness. He, too, began to faint on the sixth day of treatment and fainted repeatedly upon arising from bed for several weeks.

The laboratory observations were similar to those of the previous subject. He lost 3.4 grams of hemoglobin. Methemoglobinemia, as in Case 9, formed rapidly, averaging in the last five days, 2.0 grams per 100 cc. or 14.5

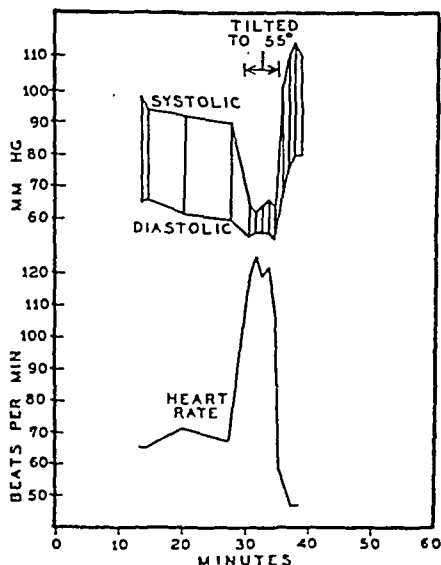


FIG. 4. CASE 7. THE FALL IN SYSTOLIC AND DIASTOLIC BLOOD PRESSURE AND THE RISE IN THE PULSE RATE UPON TILTING TO 55°, TWO MONTHS AFTER THE END OF PENTAQUINE THERAPY

per cent of the total hemoglobin. The same tests and observations were made as in Case 9. Only those yielding abnormal results will be discussed. The blood volume was not determined. Arterial and venous oxygen contents and per cent of saturation were abnormally low four weeks after the end of treatment (Table III).

The recumbent blood pressure, which was 120 mm. Hg systolic and 84 diastolic at the start of treatment, fell to 94 systolic and to 66 diastolic at the end. Hypotension, followed by syncope when the patient stood upright, was still present four months after the end of treatment (Figure 4). As in Case 9, diminished venous tone was present and persisted for three months after the end of treatment (Table IV).

Severe anorexia resulted in a weight loss of 11 kgm., which was in part regained over the next four months.

The patient was discharged from the hospital seven weeks after the end of treatment. He returned to work in the prison tailor shop and at present still complains of increased fatigability and some dizziness, but has no impotence.

He suffered a malarial relapse 14 days after the end of treatment. It was treated with quinine sulfate, responded promptly, and no further relapse occurred.

Case 10

This volunteer had symptoms similar to, but less severe than, those of the other two subjects. The recumbent blood pressure, which was 112 systolic and 58 diastolic at the start of treatment, fell to 88 systolic and 54 diastolic a month later. He had postural hypotension and several episodes of syncope; but this symptom was much less troublesome than in the other two patients. Two months after the end of the treatment period prolonged standing did not produce syncope, and the venous tone was normal.

The T-waves in the electrocardiogram diminished in amplitude. The total hemoglobin fell 3.6 grams per 100 cc.; and in addition 2.1 grams per 100 cc., or 16.7 per cent of the total hemoglobin, was converted to methemoglobin. Both during and after treatment, blood oxygen studies demonstrated arterial and venous unsaturation (Table III). No weight loss occurred. Impotence persisted for two months only.

DISCUSSION

The dosages of pentaquine administered in these studies are two or three times the dosage which has been found to be therapeutically necessary in a standardized infection (2) and are in excess of the amount that is safely tolerated in man.

There was an unexplained discrepancy between the severity of the toxicity encountered in the four groups of subjects. Possibly the discrepancies were due entirely to sampling, inasmuch as there were only five subjects in each group. The

group on 180 mgm. of base suffered very severe symptoms, which may be a reflection of the size of the daily dose. That postural hypotension and syncope did not occur in the group on 180 mgm. may be due to the fact that treatment lasted only four to eight days. Of the three groups at 120 mgm., marked methemoglobinemia, significant hemoglobin loss, and postural hypotension and syncope were limited to those subjects receiving the drug without quinine for 14 days in a therapeutic trial. The longer duration of treatment was not the only factor causing this reaction, because in the therapeutic group receiving quinine, treatment also lasted two weeks.

Of the three groups at 120 mgm. a day, the one without clinical malaria and without quinine had the least toxicity; the group with malaria but without quinine had the most severe toxicity; and the group with malaria and quinine was intermediate in the severity of their symptoms. Whether malaria tends to aggravate the toxicity of pentaquine while the concomitant administration of quinine tends to lessen it in man, contrary to its effect in experimental animals (1, 8), cannot be determined without more extensive observations.

Methemoglobinemia, hemoglobin loss, abdominal distress or pain, chest pain, anorexia, nausea and vomiting are similar to the effects of pamaquin.

The occurrence of postural hypotension and syncope suggested an analogy with the effects of sodium nitrite as described by Wilkins, Haynes and Weiss (8). The effects of nitrites are transient, while the tendency to postural hypotension in two of these subjects persisted for months. Wilkins, Haynes and Weiss demonstrated that in subjects treated with nitrites the venous tone was diminished. When nitrite-treated volunteers were tilted to 75°, diastolic and systolic arterial pressures fell, the pulse rose, and syncope ensued. Epinephrine did not prevent syncope (9).

In the subjects studied for syncope after pentaquine we found, upon tilting, a similar fall in diastolic and systolic blood pressure not observed in control cases. As in controls, and as in nitrite-treated subjects, the pulse rose. Since no observations of the venous tone in the affected subjects prior to pentaquine administration were available, comparison was made with venous tone values determined in ten control volunteers, all of whom

had also experienced an attack of malaria within the preceding several months. The two patients with severe postural hypotension had lower venous tone than the range of the ten controls. The third patient, who had recovered from hypotension and syncope when the venous tone was measured, was within the normal range. In Case 9, as in nitrite-treated subjects, epinephrine was ineffective in preventing postural hypotension and syncope.

In toxic doses, pentaquine caused the symptoms commonly recognized with toxic doses of pamaquin. In addition, pentaquine may have exerted long-lasting effects on venous tone similar to that temporarily induced by the nitrites.

The disturbance in the blood pressure regulatory mechanism observed in these three patients bears a similarity to idiopathic postural hypotension wherein the blood pressure falls when the patient stands upright because of a failure in compensatory vaso-constriction. The blood in idiopathic hypotension distributes itself in the body under the influence of gravity, as though the arterial bed were unprovided with a vaso-constriction control (10). The etiology of idiopathic orthostatic hypotension is unknown; but inability to perspire and absence of sexual potency and ejaculations point to involvement of the central sympathetic system (10, 11).

Intoxication with pentaquine similarly caused loss of ejaculatory ability in two of the three hypotensive patients. Although studies of the toxicity of pentaquine in animals have not revealed a similar effect on blood pressure, it is of interest that Moe and SeEVERS (12) have obtained evidence of central impairment of sympathetic reflexes with high doses of pamaquin in dogs. The chemical and pharmacological similarity of pentaquine to pamaquin make these observations pertinent and suggests that the abnormalities produced in man by pentaquine may be due to injury of the sympathetic centers in the central nervous system.

The cases reported are unique in that they demonstrate orthostatic hypotension due to a known etiologic agent.

Many factors have to be considered as possible explanations for the apparent anoxic anoxia, obtained in Cases 9 and 10, thirteen days after the beginning of the treatment with the drug, such as: changes in the heart or lungs; damage to the red

cells; the presence of methemoglobin or other abnormal blood pigments or a change in the oxygen combining properties of the hemoglobin.

Respiratory movements, though possibly limited by pain temporarily during treatment with pentaquine, were normal before the apparent arterial unsaturation disappeared; physical and roentgen examination of the chest failed to show evidence of obstruction, inflammatory reaction, collapse or emphysema, which might interfere with the passage of gases. The finding of a normal content of carbon dioxide in arterial and venous blood also failed to support the theory that local changes in the pulmonary tissues interfered with gaseous diffusion. Hematological observations did not disclose abnormalities in red cell size or shape which could account for poor oxygenation. Furthermore, *in vitro*, the red cells were capable of taking up oxygen.

There is a possibility that the oxygen saturation, *in vivo*, was normal but that, *in vitro*, the saturation was low because of an increase in the oxygen capacity. This state of affairs could occur in the presence of methemoglobin or other compounds containing iron in the ferric form (13). In these bloods there were appreciable amounts of methemoglobin (5 to 13 per cent of the total hemoglobin). According to Darling and Roughton (14) the reversion of methemoglobin occurs quite fast and thus produces an augmentation of the oxygen capacity.

An attempt was made to rule out this possibility by using the method of Sendroy (15, 16) for determining oxygen capacity. This method requires a shorter period for the complete oxygenation of hemoglobin and, thereby, might reduce the possibility of converting part of the methemoglobin to hemoglobin. The findings on one other patient were like the ones found by the classical method of Van Slyke used for the cases recorded in Table III.

The speed of the reconversion reaction may be too rapid for any general method now in use to exclude a reversion of the inactive pigment to the active gas combining form. Therefore, the explanation for the decrease in arterial oxygen saturation, obtained under the conditions of these studies, must await further observations.

The large arteriovenous oxygen difference (Table III) indicates that unusually large amounts

of oxygen were lost by the blood in perfusing the tissues. There was no evidence of cardiac decompensation, either on physical examination or by measurement of the venous pressure and circulation time. The stagnant anoxia, therefore, can only be explained by changes in the peripheral circulatory bed, a concept supported by the finding of a decreased venous tone.

SUMMARY

Twenty volunteers at the Illinois State Penitentiary were treated with pentaquine (SN-13,276), a new antimalarial drug related to pamaquin, in doses of 120 and 180 mgm. of the base per day. The purpose of these studies was to define the margin of safety of the drug in clinical use.

Most of the subjects experienced severe abdominal pain, nausea, anorexia, and methemoglobinemia similar to the symptoms produced by toxic doses of pamaquin.

Three subjects developed long-persistent postural hypotension and syncope, possibly due to central impairment of the sympathetic nervous system. Severe anoxia occurred during treatment and subsided over a period of weeks.

The doses used in these studies were at least double or triple the amount required for antimalarial chemotherapy.

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PENTAQUINÉ (SN-13,276), A THERAPEUTIC AGENT EFFECTIVE IN REDUCING THE RELAPSE RATE IN *VIVAX* MALARIA¹

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INTRODUCTION

Pentaquine, SN-13,276, has been found to be one of the most promising members thus far studied of a series of antimalarial drugs related to pamaquin. It has the property, also possessed by toxic doses of pamaquin (plasmochin), of eradicating *vivax* infections, accomplishing this with doses which produce only mild reactions in most individuals. This paper describes the therapeutic trials upon which these conclusions are founded.

Pentaquine, (5-isopropylaminoamylamino)-6-methoxy-quinoline, differs constitutionally from pamaquin in the structure of the aliphatic side chain in the 8-position (Figure 1). The drug was supplied³ and used in the form of the monophosphate salt which is 75.5 per cent base. All dosages recorded in this paper are in terms of the base

weight in order to facilitate comparison with pamaquin.

In the experimental animals, the antimalarial effect of pentaquine, its pharmacology and toxicology have been investigated (1, 2). Activity, 80 to 128 times that of quinine and two to eight times that of pamaquin in avian malaria, first focused attention on this compound.

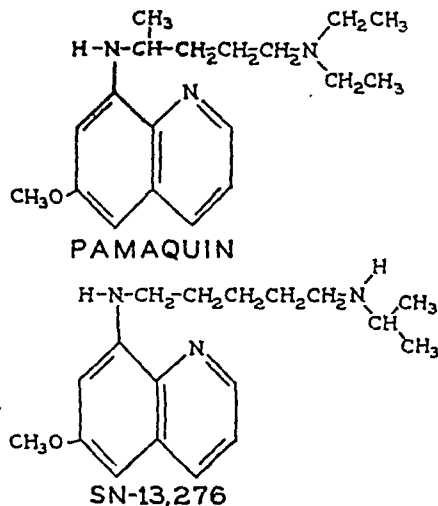


FIG. 1. PENTAQUINÉ (SN-13,276) AND PAMAQUIN (PLASMOCHIN) ARE 6-METHOXY-8-AMINOQUINOLINES DIFFERING ONLY IN THE STRUCTURE OF THE ALIPHATIC SIDE CHAIN IN THE 8-POSITION

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. This work was further aided by the participation of Army Medical officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

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³ Pentaquine was synthesized by Dr. Nathan Drake, Department of Chemistry, University of Maryland.

In mammals it is rapidly absorbed from the gastro-intestinal tract. Maximum plasma levels usually proportional to the dose are attained in one and a half to two hours, little or none remaining after six or eight hours. Tissue storage apparently does not occur, and urinary excretion is negligible. Disappearance of the drug is probably due to rapid degradation in the animal body. In mammals its physiological disposition, therefore, is similar to that of pamaquin, the only difference being that plasma concentrations are sustained longer with pentaquine.

With certain exceptions the toxicity of pentaquine in experimental animals is similar qualitatively to that of pamaquin. In acute, and in short-term chronic toxicity studies, pentaquine was from one-fourth to one-half as toxic as pamaquin. In the dog, pamaquin in large doses produces severe anorexia, emaciation and ocular paralysis due to central impairment of the sympathetic innervation of the eye. In high dosages pamaquin produces leukopenia, neutropenia, anemia, methemoglobinemia, emaciation, depression, and liver damage in the monkey, effects which are not produced with pentaquine in this species. On the other hand, in both dog and monkey an ill-defined deleterious effect on the heart and circulation appears to be more severe with toxic doses of pentaquine than with pamaquin.

The concurrent administration of quinine increases the severity of the toxicity of pentaquine in both the rat and monkey but does not alter the toxicity qualitatively. In this respect, pentaquine behaves like pamaquin in the experimental animal.

PROCEDURES AND METHODS

Details of the drug-testing procedures are reported elsewhere (3). In brief, healthy white inmate volunteers⁴ at the Illinois State Penitentiary were inoculated with Southwest Pacific (Chesson) *vivax* malaria by the bites of infected mosquitoes. Uniformity of the disease was sought by the use of presumably susceptible individuals in primary attacks or first or second relapses, after inoculation with the bites of ten mosquitoes whose infectivity was verified by dissection. None of the subjects had had prolonged suppressive treatment, and therapy with test drugs was begun early in the clinical attack. Accidental reinfection was not a problem because the penitentiary was in a non-endemic area.

In spite of these efforts to secure uniformity in the induced disease, variation became apparent. It was possible, however, to divide the more severely from the less severely infected on the basis of the length of the prepatent and preceding latent periods (4). The less severely infected subjects, who had long prepatent or latent periods, were characterized by a relapse rate of 67 per cent after treatment with so-called suppressive drugs, while the more severely infected subjects had a relapse rate of 98 per cent. A small third group of volunteers had massive infections, being inoculated by the bites of 80 infected mosquitoes, instead of the ten used in the standard procedure, or by the intracutaneous injection of

the dissected salivary glands of 45 to 75 infected mosquitoes. The relapse rate of this group was 100 per cent after treatment with suppressive drugs. The subjects accordingly were divided into three categories representing (1) a moderate, (2) a severe, and (3) an extraordinarily severe challenge to the therapeutic efficacy of the test drug. Pentaquine was administered to 82 subjects in doses of 60 mgm. of base or less, daily. An additional group of 17 subjects without clinical malaria, treated during latency, were included in toxicity studies. A total of 99 subjects are accordingly available for this report.

The patients were hospitalized during treatment but were allowed to be up *ad libitum*. Symptoms were elicited daily or oftener, evidence of toxicity was collected, and the effect on the disease evaluated by following the fever and parasitemia. Blood films for parasite counts were made daily during the clinical disease and every two days thereafter until relapse occurred. If no relapse occurred in six months, films were then made once weekly. Some of the subjects received quinine or paludrine concurrently with pentaquine. All drugs were given in oral doses every four hours for 14 days, treatment being started after the subject had been febrile for one day and had had parasitemia for two.

The concentration of pentaquine in the plasma was estimated every other day using the modified method (5) of Brodie, Udenfriend and Taggart (6).

The amounts of methemoglobin and hemoglobin were determined daily on venous blood by the photocolormetric method of Wendel (7).

RESULTS

Therapeutic effect

Suppression of fever and parasitemia. When administered alone in doses of 30 or 60 mgm. of base a day, pentaquine resulted in the disappearance of fever in two to four days and of parasitemia in three to six (Table I). Its effect in this respect was not significantly different from the effect of quinine alone or of quinine and pentaquine administered concurrently.

Radical cure. Twenty-six *moderately* infected subjects received 60 mgm. of the base daily and four others similarly infected received 30 to 45 mgm. In these 30 subjects, quinine was concurrently administered. Only one suffered a relapse. The relapse rate of 3 per cent thus obtained may be compared to the 67 per cent obtained when similar subjects were treated with suppressive drugs (Table I).

Of 21 volunteers with *severe* infections treated with or without quinine at doses of pentaquine ranging from 15 to 45 mgm. of the base a day, 20

⁴ The studies reported in this paper would not have been possible except for the enthusiastic cooperation of the inmate volunteers and administrative officials of Stateville Penitentiary.

TABLE I

Therapeutic effect of pentaquine administered for 14 days in mosquito-induced vivax malaria (Chesson)

Daily dosage (base)	No. of subjects	Mean concentration of drug in plasma		First afebrile day		First day without parasitemia		Relapse ratio individuals relapsed/ individuals treated			Duration of subsequent latent period in subjects who relapsed	Duration of follow-up period in subjects who did not relapse
		Range	Mean	Range	Mean	Range	Mean	Moderate infection	Severe infection	Massive infection		
mgm. 15 with quinine	5	gamma/l. 30-51	gamma/l. 40	day 2-3	day 2.6	day 3-4	day 3.6		5/5		days 9, 12, 15, 21, 37	
30	5	25-48	34	2-4	2.6	4-6	4.6		5/5		3, 4, 4, 9, 40	
30 with quinine	10	12-67	44	1-3	1.9	3-5	4.1	0/1	8/9		11, 12, 17, 38, 41, 41, 48, 64	6 months
45 with quinine	5	24-58	43	2	2.0	3-5	4.0	0/3	2/2		14, 15	
60	5	29-49	41	2-4	2.8	3-5	4.0		2/4	1/1	12, 128; 17	9 months
60 with paludrine	5	139-452	270	1-4	2.4	4-7	4.8		4/5		24, 29, 36, 120	4 months
60 with quinine	47	26-196	63	0-4	2.0	1-6	3.6	1/26	3/17	4/4	22, 45, 54, 70; 19, 43, 44 87	See notes below
Controls Quinine alone	13			1-4	2.3	4-6	4.7	4/5	6/6	2/2	10, 15, 25, 221; 7, 8, 10, 11, 11, 12; 9, 10	12 months
Other suppressive drugs	80							8/12	44/45	4/4		4-15 months

The dosages of pentaquine are in terms of the base. Quinine was administered in doses of 2.0 grams of the sulphate daily and paludrine in doses of 1.0 gram of the base daily. Drugs were administered on a four-hour schedule. The plasma concentrations are the average of seven estimations for each patient; blood for plasma analyses was collected about one hour after the 8 a.m. dose every other day. The first afebrile day and the first day without parasitemia are counted from the day on which the treatment was begun, that day being day 0. The subjects treated with pentaquine at 60 mgm. daily concurrently with quinine who have not relapsed have been observed for varying periods of time, as follows: *moderately infected*—eight for five or six months, 11 for 15 or 16 months and six for 14 months or more; *severely infected*—nine for 21 to 24 months, one for 16 months, four for 14 months or more. A longer period of observation is unlikely to change these results materially because in 107 cases which relapsed after treatment with 8-aminoquinolines and quinine over 90 per cent relapsed within six weeks. The 75 controls (4) were treated with one or more of the following drugs: SN-4,095, SN-8,617, SN-10,275, SN-11,437 (metachloridine), SN-7,618 (chloroquine), quinine, quinacrine (atabrine), and paludrine (1).

relapsed. This relapse rate of 95 per cent is comparable to the 98 per cent relapse rate obtained when similar severely infected subjects were treated with suppressive drugs.

Dosages of pentaquine of 60 mgm. of the base a day, alone, with paludrine, or with quinine were effective in modifying the relapse rate of the *severely* infected patients. Given alone, pentaquine therapy decreased the relapse rate to two relapses

for the four subjects; given with paludrine, it did not lower the relapse rate further; given with quinine, however, pentaquine reduced the relapse rate of the severely infected subjects to three relapses out of 17 subjects treated. Therefore, the concurrent administration of quinine with pentaquine was clearly more efficacious than pentaquine alone or the combination of pentaquine with paludrine. The quinine-pentaquine regime at the

dosage of 60 mgm. a day of the latter drug resulted in a reduction of the relapse rate from 98 per cent to 18 per cent in subjects with severe infections.

In subjects treated in primary attacks after *extraordinarily heavy* inocula, however, the 100 per cent relapse rate obtained after suppressive drugs was unchanged. Four subjects were treated with pentaquine at 60 mgm. and quinine; all relapsed.

Concentration of pentaquine in plasma

It will be observed that the mean concentrations of drug in the plasma varied widely (Table I). The individual variations were so great that the group means have little significance. The increase in plasma concentrations attained when quinine was concurrently administered with pentaquine may not be significant because of the wide scatter. There was only a slight positive correlation between dosage of pentaquine and plasma pentaquine concentration on regimes in which quinine was concurrently administered. A striking

increase in plasma concentration of pentaquine when paludrine was concurrently administered, on the other hand, was uniformly obtained.

The therapeutic effect of pentaquine, however, was not enhanced by the concurrent administration of paludrine in spite of the increased plasma concentrations. Furthermore, in any one dosage regime there did not appear to be a correlation between the individual plasma concentrations and the therapeutic results.

Toxicity

Ninety-nine white subjects were treated with pentaquine alone or in combination with paludrine or quinine in doses not exceeding 60 mgm. base a day. Symptoms elicited were qualitatively like those produced by pamaquin, but less severe than would occur with equal doses of the latter drug.

Toxicity at 15 to 45 mgm. of base per day. Except for the production of small amounts of methemoglobin no significant symptoms occurred in the

TABLE II
The toxicity of pentaquine given for 14 days in doses not exceeding 60 mgm. of the base daily

Daily dose of SN-13,276 (base)	Number of cases	Symptoms			Laboratory observations				Approximate daily dose of pamaquin which produces comparable toxicity
		Abdominal discomfort		Anorexia or nausea	Fever over 100.6° rectal	Leucocytosis (over 12,000 per cu. mm.)	Methemoglobin formed*		
		Moderate	Severe	Moderate			Range	Mean	
<i>mgm.</i> 15 with quinine	<i>no.</i> 5	<i>no.</i>	<i>no.</i>	<i>no.</i>	<i>no.</i> 1	<i>no.</i> 1	<i>per cent of total hgb.</i> 2.1-3.3 2.6		<i>mgm.</i> Less than 15
30	5	1					2.7-3.8	3.2	15
30 with quinine	10					2	1.0-4.9	2.8	15
45 with quinine	5	1				1	2.1-3.3	2.6	15
60	5	1					3.6-7.0	4.5	30
60 with paludrine	5	1	2				2.8-8.5	4.5	30
60 with quinine	64	27	1	3	3	14	0.8-10.2	3.9	30

* Methemoglobin values are the average of the last five days of treatment. The average value obtained on analysis of the blood of 196 normal subjects was 1.8 per cent of total hemoglobin. The standard deviation was 1.10 and the standard error of the mean 0.08.

25 subjects receiving doses of 45 mgm. or less daily (Table II). One patient had transient unexplained fever on the ninth day of treatment. Three patients had transient mild leukocytosis. The degree of methemoglobinemia and the symptomatology were similar to that produced by a daily dose of 15 mgm. of pamaquin base.

Toxicity at 60 mgm. of base per day. With doses of 60 mgm. of pentaquine base, toxic symptoms occurred. In no instance among 74 subjects were the symptoms severe enough to warrant discontinuance of the drug (Tables II and III).

Abdominal discomfort was the outstanding symptom. Twenty-nine subjects complained of pain, either mild but persistent or moderately severe and transient. Fifteen others noted mild,

transient abdominal discomfort. In one subject pain was severe. The distress was usually epigastric, sometimes radiating into the retrosternal area. It bore no apparent relationship to any bodily function and was unrelieved by food. In order not to mask the symptomatology, analgesics were withheld. Occasionally epigastric tenderness was an accompanying finding.

Mild anorexia was a common occurrence. In a few subjects, it was severe enough to result in a small weight loss which was, however, quickly regained when the therapeutic course was completed.

A few volunteers complained of transient weakness, headache, or diarrhea, but these symptoms were not severe. In three subjects, fever on the fourth, seventh, or tenth day was observed.

TABLE III

The therapeutic and toxic effects of pentaquine administered at a dose of 60 mgm. of the base daily, with quinine for 14 days

Case number	Severity of infection	Mean concentration of SN-13,276 in plasma	Result	Symptoms		Laboratory observations			Comment*
				Abdominal discomfort*	Anorexia and nausea*	White blood count*	Met-hemoglobin formed†	Change of hemoglobin‡	
		gamma/L.				per cu. mm.	per cent of total hemoglobin	grams per 100 cc.	
1	No clinical malaria (treated during latency)	47	Cases 1 through 17 were treated after several months of spontaneous or induced latency. Many had had several relapses and had developed a considerable degree of immunity; observations on these patients are of value only for toxicity. None have relapsed during many months of observation.	0	0	N	2.7	+0.6	Pain in chest ++
2		103		++	++	18,500	6.1	-0.1	
3		66		0	0	N	6.1	-0.4	
4		63		++	0	N	2.9	-0.1	
5		54		++	0	N	5.9	-0.9	
6		39		++	0	12,000	2.7	-1.2	
7		107		+	+	13,700	6.6	-0.3	
8		117		++	+	13,700	9.8	-2.1	
9		42		0	+	14,400	3.5	-1.1	Headache +; fever 5th and 6th days
10		39		++	0	N	4.1	-1.3	Headache +
11		20		++	+	N	2.1	-1.1	Diarrhea +
12		49		++	0	13,100	3.1	-1.6	Headache +
13		35		++	+	N	3.8	-1.3	Diarrhea +
14		158		0	0	N	8.4	-1.6	
15		58		++	+	13,100	3.0	-1.5	
16		40		0	0	—	3.9	-1.0	
17		25		0	0	—	1.4	-1.3	
18	Moderate (long prepatent or preceding latent period)	114	No relapse in 6 mos.	+	0	N	5.7	-1.4	Fever on 7th day
19		49	No relapse in 6 mos.	++	0	N	5.2	-0.4	
20		64	No relapse in 6 mos.	++	0	N	5.0	-0.5	
21		45	No relapse in 5 mos.	0	0	N	2.9	-0.7	Diarrhea + Chest pain ++
22		37	No relapse in 5 mos.	+	+	N	2.5	-0.5	
23		41	No relapse in 5 mos.	0	+	N	2.7	-0.1	
24		28	No relapse in 5 mos.	0	0	N	1.4	+0.3	
25		63	No relapse in 5 mos.	++	0	16,200	4.3	-0.5	
26		43	No relapse in 5 mos.	0	0	N	2.9	-0.3	
27		165	No relapse in 4 mos.	+	0	3,600-15,500	5.9	-0.6	
28		45	No relapse in 4 mos.	0	0	N	4.8	+0.4	Headache +; diarrhea +
29		96	No relapse in 4 mos.	0	0	N	8.3	-1.0	
30		80	No relapse in 4 mos.	+	0	—	3.6	+1.0	

TABLE III—Continued

Case number	Severity of infection	Mean concentration of SN-13,276 in plasma	Result	Symptoms		Laboratory observations			Comment*
				Abdominal discomfort*	Anorexia and nausea*	White blood count*	Met-hemoglobin formed†	Change of hemoglobin‡	
		gamma/L.				per cu. mm.	per cent of total hemoglobin	grams per 100 cc.	
31	Moderate (long prepatent or preceding latent period) continued	39	No relapse in 4 mos.	0	+	—	3.1	—0.5	Headache +
32		39	No relapse in 4 mos.	+	0	12,300	2.5	—0.5	
33		82	No relapse in 4 mos.	0	0	N	2.1	—1.1	
34		37	No relapse in 3½ mos.	0	++	N	3.2	+0.1	
35		74	No relapse in 3½ mos.	++	0	N	4.6	—0.6	
36		44	No relapse in 3½ mos.	+	0	N	2.2	—0.3	
37		37	No relapse in 2½ mos.	++	0	N	3.1	—0.9	
38		70	No relapse in 2½ mos.	+	0	N	1.6	—0.6	
39		50	No relapse in 2½ mos.	0	0	N	2.8	—0.7	
40		27	No relapse in 2 mos.	0	0	15,600	1.3	—0.9	
41		54	No relapse in 2 mos.	+	0	N	3.8	—1.5	
42		68	No relapse in 2½ mos.	++	0	N	3.9	—1.3	
43		30	Relapsed in 22 days	0	0	N	2.8	—1.5	
44	Severe (short prepatent or preceding latent period)	56	No relapse in 12 mos.	++	0	N	3.9	+0.5	Headache + Headache + Diarrhea + Headache + Diarrhea +; weakness +
45		55	No relapse in 12 mos.	0	0	N	4.6	—1.0	
46		68	No relapse in 12 mos.	0	0	N	9.9	—2.0	
47		87	No relapse in 12 mos.	++	0	N	10.2	—1.6	
48		57	No relapse in 11 mos.	++	+	—	3.3	+0.3	
49		130	No relapse in 9 mos.	++	0	N	6.4	—0.7	
50		59	No relapse in 9 mos.	++	0	N	4.3	—1.5	Fever on 4th day Headache + Weakness ++
51		68	No relapse in 9 mos.	++	0	N	3.4	0.0	
52		90	No relapse in 9 mos.	0	0	12,000	5.5	—0.1	
53		54	No relapse in 4 mos.	++	+	N	2.9	—0.6	
54		54	Relapsed in 70 days	+	0	13,600	0.8	—0.9	
55		196	No relapse in 2½ mos.	+++	++	N	2.7	—0.2	
56		64	No relapse in 2½ mos.	++	+	N	1.2	—1.1	Headache + Weakness ++
57		43	Relapsed in 45 days	0	0	12,800	1.0	—1.1	
58		34	Relapsed in 54 days	++	0	N	1.1	+0.1	
59		26	No relapse in 2 mos.	++	+	N	4.1	—0.8	
60		27	No relapse in 2 mos.	0	0	N	1.3	+0.2	
61	Massive (intracutaneous inoculation of glands of 45-75 mosquitoes or 80 bites)	54	Relapsed in 87 days	0	0	N	2.4	—1.4	Afebrile relapse followed by spontaneous latency
62		38	Relapsed in 19 days	++	0	N	2.9	—0.1	
63		68	Relapsed in 43 days	0	0	N	5.4	—1.7	
64		93	Relapsed in 44 days	+	0	N	4.1	—0.3	

* The clinical impression of the severity of the toxic symptom is graded on the basis of + to +++++. N represents a normal leucocyte count.

† Methemoglobin values are the average of the findings on the last five days of treatment. The average obtained on analysis of the blood of 196 normal subjects was 1.8 per cent of total hemoglobin. The standard deviation was 1.10 and the standard error of the mean 0.08.

‡ The change of hemoglobin was the difference between the average of the hemoglobin values of the first five days of treatment and the average of hemoglobin estimations on the last and four subsequent days.

Electrocardiograms revealed a diminution in the height of the T waves in some or all of the leads. T₃ sometimes became inverted; in other cases an inverted T₃ became upright. In only a few instances was the T wave amplitude reduced below normal height. Serial electrocardiograms in such cases showed a return to the configuration of the control tracing after the course of treatment was

completed. Urinary abnormalities were not observed; the urinary output remained normal.

There was an elevation of the leucocyte count above 12,000 per cu. mm. in 14 subjects. Three subjects had a leucopenia as low as 3000 to 3600. However, in all instances of either leucocytosis or leucopenia, differential leucocyte counts were normal, and the abnormalities were in most in-

stances unconfirmed by serial leucocyte estimations.

In the presence of clinical malaria, it is difficult to evaluate a slight fall in hemoglobin. However, in this series, 16 of the 17 subjects receiving pentaquine at 60 mgm. of pentaquine base a day with quinine for 14 days, at a time when they had neither fever nor parasitemia, demonstrated a small decline in hemoglobin. The initial and terminal hemoglobin values were the average of five daily determinations. The loss amounted to an average of 1.0 gram per 100 cc. of blood (6.7 per cent of the initial hemoglobin) with a range of + 0.6 to - 2.1 gram per 100 cc. Although the loss was small, it exceeded one gram in 11 of the 17 subjects. No subject developed acute hemolytic anemia.

The production of methemoglobin was common and, in effect, further reduced the amount of available oxygen-carrying hemoglobin. In the 17 subjects without malaria, methemoglobinemia averaged 0.66 gram per 100 cc. (4.4 per cent of the total hemoglobin) during the last five days of treatment. If this be added to the 1.0 gram per cent of hemoglobin lost, the total diminution in oxygen-carrying hemoglobin amounted to 1.66 grams per 100 cc. of blood or 11.1 per cent of the total hemoglobin.

Of the 74 subjects treated with 60 mgm. of the base daily, only nine failed to show methemoglobinemia in excess of the mean value (1.8 per cent of the hemoglobin) obtained in normal blood by this method. In ten subjects, it exceeded 6 per cent of the total hemoglobin, above which level cyanosis was clinically evident. In the remaining 55, methemoglobin was produced but not in amounts great enough to be detected on inspection.

Comparison with pamaquin. The average per cent of hemoglobin converted to methemoglobin bears a rough relationship to the clinical symptomatology and permits quantitative comparison with the various pamaquin regimes. The toxicity of pentaquine at 15 to 45 mgm. of base per day was equivalent to no more than that of 15 mgm. of pamaquin base. At 60 mgm. of pentaquine base, either alone or in conjunction with paludrine or quinine, the toxicity was approximately equivalent to that of 30 mgm. of pamaquin base (67 mgm. of pamaquine naphthoate).

Effect of concurrent administration of quinine on toxicity. The toxicity of pentaquine in this series of 99 cases was not increased by the concomitant administration of quinine, as it is in experimental animals. In fact, average methemoglobin values were slightly lower for the groups receiving quinine (Table II). However, the number of men treated without quinine was too small to permit final evaluation.

DISCUSSION

A clinical trial of pentaquine in 99 white subjects infected with *vivax* malaria has proved this drug to be a promising antimalarial agent. In doses which were well tolerated, the drug caused a marked reduction in the relapse rate when administered in conjunction with quinine. In a group of moderately infected patients the relapse rate was reduced from 67 per cent to 4 per cent and in severely infected patients, from 98 per cent to 18 per cent. In subjects with massive inoculations it did not prevent relapse (Figure 2).

The effectiveness of pentaquine at 60 mgm. of the base in preventing relapse was enhanced by the concurrent administration of quinine. This finding is paralleled by observations previously made (8 to 11) with pamaquin and quinine. It is of interest that in the severely infected group in which quinine alone has a negligible effect and pentaquine and pamaquin alone only a moderate effect in preventing relapse, the concurrent administration of quinine with either of these drugs is highly effective. Quinine appears, therefore, to potentiate the effect of these two 8-aminoquinolines. Synergism between pentaquine and paludrine, however, could not be demonstrated in severely infected subjects.

Pentaquine has clear therapeutic superiority over the usual suppressive antimalarials. It shares this superiority with pamaquin which also has been demonstrated (8 to 11) to have curative properties in *vivax* malaria. With both drugs the limiting factor is the inherent toxicity of the therapeutic agent. Sixty mgm. of pentaquine base have an effect on the relapse rate approaching that of 90 mgm. of pamaquin base. Ninety mgm. of pamaquin, however, cause severe toxicity, making it impractical for clinical use. Sixty mgm. of pentaquine, on the other hand, causes toxicity sim-

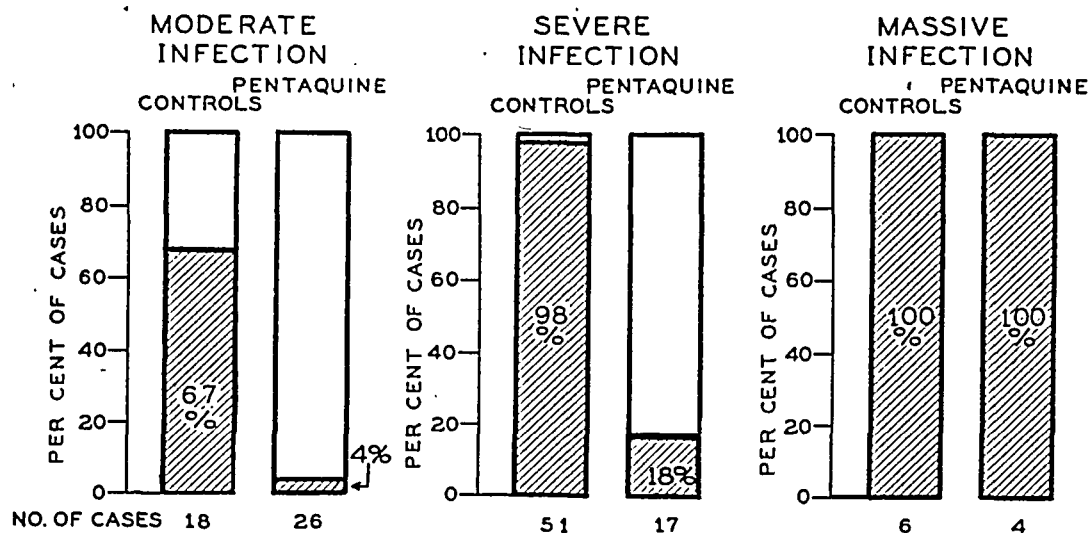


FIG. 2. THE EFFECT OF PENTAQUINE ON THE RELAPSE RATE OF MOSQUITO-INDUCED *VIVAX* MALARIA (CHESSON STRAIN)

Pentaquine was given for two weeks at 60 mgm. of the base per day. Quinine was concurrently administered. Control subjects were treated with a variety of suppressive drugs. Per cent figures indicate relapse rate.

ilar to that produced by 30 mgm. of pamaquin, a dosage well tolerated in most white patients.

Pentaquine is not, however, an innocuous drug. Studies of its effect at 120 and 180 mgm. of base daily have shown serious toxicity including, in addition to more severe symptoms of the type described at 60 mgm. base, severe weakness, prostration, anoxia, postural hypotension and syncope persisting for months after the termination of therapy (12). The potential occurrence of severe symptoms at lower doses must be borne in mind when less robust or smaller individuals are treated.

The absence of acute hemolytic anemia in this series may be due in part to sampling and in part to limiting studies to white subjects. With pamaquin, hemolytic anemia occurs rarely in whites, and more commonly in colored races (2). Pentaquine has in fact precipitated acute hemolytic anemia in a white individual (2, 13).

Pentaquine may prove clinically useful in doses less than 60 mgm. a day. Although pamaquin must be given at 90 mgm. of base to cure the severely infected non-immune individual (10, 11), doses as low as 27 mgm. of base a day lowered the relapse rate to a striking degree in returned service men who had been on prolonged suppressive therapy (14). By analogy it may be that pentaquine will be found useful in doses considerably lower than those required for the severely infected

volunteers studied during this investigation. At doses lower than 60 mgm. we have found negligible toxicity, but the possibility of acute hemolytic anemia must not be overlooked. We believe hospitalization throughout the treatment course for the purpose of observation (as well as to ensure the four-hourly administration of the drug) to be essential in view of our present limited knowledge of this new therapeutic agent.

CONCLUSIONS

The therapeutic effect of pentaquine (SN-13,276) has been studied in acute attacks of Chesson Southwest Pacific strain of *vivax* malaria, under standardized conditions.

Pentaquine is effective in reducing the relapse rate of *vivax* infections. Its curative properties are enhanced by the concurrent administration of quinine. A daily dose of 60 mgm. of pentaquine base (80 mgm. of the monophosphate) and 2 grams of quinine sulfate, administered concurrently in divided doses every four hours for 14 days, reduced the relapse rate in severely infected patients from 98 per cent to 18 per cent. In moderate infections, the relapse rate was reduced from 67 per cent to 4 per cent. With massive infections, however, combined pentaquine-quinine therapy failed to prevent relapse in the four subjects studied.

Some evidence suggests that considerably lower doses of pentaquine may effect a radical reduction of relapse rate of *vivax* malaria in individuals who stop suppressive therapy upon return to non-endemic areas after long residence in hyper-endemic regions.

Pentaquine should only be administered under close medical supervision, preferably during hospitalization. The daily dose of 60 mgm. base should not be exceeded. This dose has approximately the same toxicity as 30 mgm. of pamaquin (base) or 67 mgm. of its naphthoate salt. The toxicity of pentaquine is too great to warrant its use in prophylaxis or prolonged suppression of malaria. The safe therapeutic dose for children and the toxicity of the drug in negroes and individuals of mixed racial extraction is at present undetermined.

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THE CLINICAL TRIAL OF EIGHTEEN ANALOGUES OF PAMAQUIN (PLASMOCHIN) IN *VIVAX* MALARIA (CHESSON STRAIN)¹

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In the malarial research program conducted on a national scale during World War II, several drugs were developed which had marked superiority over quinacrine (atabrine) and quinine in their ability to terminate individual attacks of *vivax* malaria (1). For example, chloroquine (SN-7618) in terms of oral dosage and the resulting plasma concentration can be administered in amounts many times that required to suppress the disease (2). The margin between the therapeutic and toxic dose of chloroquine is several times that which exists for quinine and quinacrine. When these new compounds, however, failed, even at high dosages, to affect the relapse rate it seemed doubtful that further extension of research aimed chiefly towards finding a more effective and less toxic, and primarily suppressive, drug would lead

to early development of a compound that would effect a radical cure.

Pamaquin (plasmochin) has been recognized for many years as an antimalarial drug with unusual properties (3 to 9). It has proved more efficacious in reducing the relapse rate of *vivax* malaria than previously used drugs. Because of its toxicity, however, pamaquin fell into disrepute and its use in the Armed Services of the United States was discontinued (10).

The most direct approach to the problem was to explore the chemical analogues of pamaquin in the hope of developing a compound which possessed the therapeutic properties of pamaquin but which was free of its undesirable toxic effects. Consequently, in 1944, the national program was realigned, the major emphasis being placed on pamaquin analogues. The chemical structure of pamaquin is shown in Figure 1. It is a member

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The studies were planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for the Coordination of Malarial Studies. This work was further aided by the participation of Army Medical officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

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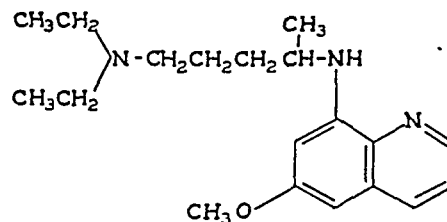


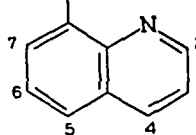
FIG. 1. STRUCTURAL FORMULA OF PAMAQUIN (PLASMOCHIN)

of a class of compounds in which there is a substituted aminoalkyl group on an 8-amino radical on a quinoline nucleus (Figure in Table I). Pamaquin and 21 other substituted 8-aminoquinolines have been studied clinically in human volunteers.

METHODS AND PROCEDURES

General. Details of the general procedure and the plan of observation are reported elsewhere (11). Healthy, presumably susceptible white volunteers at Stateville

TABLE I

Survey number	General formula of 8-(ω-aminoalkylamino) quinolines			Antimalarial activity in avian infections (1) (quinine equivalent)		Short term chronic toxicity in mammals (1) (pamaquin equivalent)	
	<div><div>$\begin{array}{c} \text{R}' \\ \diagdown \\ \text{N} - (\text{CH}_2)_n - \text{NH} \\ \diagup \\ \text{R}'' \end{array}$</div><div></div></div>			Gallina- ceum in the chick	Lophurae in the duck	Rat	Monkey
	$\begin{array}{c} \text{R}' \\ \diagdown \\ \text{N} - \\ \diagup \\ \text{R}'' \end{array}$	$-(\text{CH}_2)_n-$	Nuclear substituents				
SN-1,452	amino	n-propyl		40	3		
SN-12,352		n-hexyl	6-methoxy	20	6	0.5	0.25+
SN-12,354			5,6-dimethoxy	15-60	20	0.6	<0.25
SN-12,451	ethylamino		6-methoxy	30	80	0.4	0.25+
SN-13,276	i-propylamino	n-amyl	6-methoxy	100	150	—	0.25
DR-15,302		1-methylbutyl	4-methyl, 6-methoxy	—	256	—	—
SN-9,972			5,6-dimethoxy	80	80	—	1
SN-13,274	6-methoxy			20	40	—	0.5
SN-13,232		n-hexyl	40	80	—	0.5	
SN-13,429		1-methylbutyl	80	80	0.3	0.5	
SN-13,233	n-propylamino	n-hexyl	6-methoxy	30	100	0.4	0.5
SN-13,380	n-butylamino		20	40	0.2	0.5	
SN-191	diethylamino		1-methylpropyl	6-hydroxy	20	30	—
SN-971		1-methylbutyl	6-methoxy	10-40	80	1	1
SN-8,233			5,6-dimethoxy	40	200	—	—
SN-11,191			6-methoxy	30	100	0.4	0.5
SN-13,619		n-hexyl	2-methyl	1.5	3	—	0.25
SN-13,697			6-hydroxy	—	2	—	<0.25
SN-13,694			5-chloro; 6-methoxy	3	10	—	0.5
SN-14,011			4-methyl; 6-methoxy	10	600	—	—
SN-11,226	diethylaminopropylamino	n-propyl	6-methoxy	2	<3	—	—
SN-12,325	methyl i-propyl amino	n-hexyl	6-methoxy	8-60	60	—	0.5

Penitentiary,³ which is located in a non-endemic area, were infected with Southwest Pacific virus malaria (Chesson strain) (12) by the bites of infected *Anopheles*

³ The investigations reported in this paper would not have been possible except for the enthusiastic cooperation of the inmates and the administrative staff of Stateville Penitentiary.

quadrinaculatus mosquitoes. This strain is characterized by a high relapse rate when treated with non-curative drugs such as quinine and quinacrine, by a short period of latency between successive attacks, and by almost complete absence of delayed primary attacks (13, 14). Volunteers undergoing primary attacks and first or second relapses served as test subjects. Treatment was initiated promptly after the appearance of fever and para-

sitemia in order to minimize the effects of acquired immunity. Five volunteers composed a test group in the standard procedure. When a compound showed promise, however, studies were expanded to larger groups and different dosages.

Selection of drugs. Only pamaquin analogues with pamaquin-type toxicity in animals were used; no plasmascid type compounds received trial in man. As new drugs were synthesized, they were tested in avian infections for antimalarial activity and in rodents, dogs, and monkeys for toxicity. On the basis of the animal experiments, the drugs that offered promise received clinical testing in man. Table I summarizes the chemical structure, avian antimalarial activity and mammalian toxicity of the drugs reported in this paper. They were all 8-(ω -aminoalkyl-amino) quinolines, differing from each other in nuclear substituents, length and character of the inter-nitrogen aliphatic chain, and the number and nature of substituents on the terminal amine nitrogen.

Dosage. Early in the course of the drug-testing program it had been planned to conduct the human clinical trials at approximately the maximum tolerated dose as estimated from the animal experiments. However, severe toxic reactions resulted from the use of four of these drugs⁴ at this high dosage and this approach was abandoned. Pamaquin is therapeutically efficacious at a daily dose of 90 mgm. of base (equivalent to 200 mgm. of the naphthoate) and is well tolerated at a dose of 15 mgm. of base per day. Since a new drug, to be ideal, should possess at least the therapeutic efficacy of pamaquin at 90 mgm. (base) and no greater toxicity than that of pamaquin at 15 mgm. (base), it was decided to test new drugs at 15/90, or 1/6, of their maximum tolerated dose as estimated from monkey experiments. If, at this dosage, human toxicity was found to be absent or mild, the drugs were tested at a higher dosage level.

Administration of drugs. The drugs were administered over a 14-day period in equal doses every four hours to insure fairly constant concentrations in the body fluids. Quinine was administered concurrently with the drugs for two reasons: (1) the synergistic effect of quinine on pamaquin may also extend to pamaquin analogues, and (2) if it is assumed that a curative agent may prevent relapse by action chiefly on hypothetical exo-erythrocytic stages of the parasite with little or no action on the erythrocytic stages, the concurrent administration of an anti-trophozoite agent is indicated. Qui-

nine was given as the dihydrochloride or sulfate in doses of 2.0 grams of the salt daily, in six equally divided doses at four-hour intervals.

Classification of cases. It has been shown elsewhere (14) that under the standardized conditions of this investigation, the relapse rate of the Chesson strain is high when suppressive drugs are used. Some individuals, however, fail to relapse although treated with drug regimes ordinarily considered to possess only suppressive activity. It has been possible to relate probability of relapse with the length of the prepatent and latent periods (14). The relapse rate after treatment of attacks in patients who had prepatent periods less than 15 days or latent intervals less than 30 days was 98 per cent, whereas it was only 67 per cent when the prepatent or latent periods were longer. On the basis of length of prepatent and latent periods, therefore, this series of cases has been divided into two groups. One group consists of individuals whose prepatent periods were 14 days or less and whose preceding latent intervals were 29 days or less. This group offers a more severe therapeutic test for an antimalarial agent than the remainder of the cases. The latter group, with longer prepatent and latent periods, offers only a moderate therapeutic test for an antimalarial drug, because of lower probability of relapse.

Chemical estimation of drugs in the plasma. Blood was drawn for analysis every other day during the 14-day course of treatment and for four days thereafter.

Oxalated whole blood was centrifuged for 15 minutes at 2,000 r.p.m., the plasma was separated and re-centrifuged for 60 minutes at the same speed to insure complete removal of the components of the buffy coat.

Quinine was estimated by the method of Brodie and Udenfriend (15). The 8-aminoquinolines were estimated by the method of Brodie, Udenfriend and Taggart (16) modified as follows: 20 ml. of heptane and 0.5 ml. of iso-butyl alcohol were placed in a 60-ml. glass stoppered bottle. Ten ml. of plasma and 10 ml. of 0.1 N NaOH were added and the mixture shaken for 10 minutes. The mixture was then transferred to a 50-ml. centrifuge tube and centrifuged for five minutes. The water phase was aspirated and 15 ml. of the heptane phase was transferred to a 40-ml. glass-stoppered pointed centrifuge tube containing 0.5 ml. of coupling reagent (diazotized sulphanilic acid). The mixture was then shaken for five minutes and centrifuged. The heptane layer was removed by aspiration. Not less than 0.3 ml. of the water layer was transferred to a special microcuvette and the transmission read in a spectrophotometer (Coleman) at a wave length of 480 millimicra.

Average plasma concentrations of quinine and test drug were arrived at by calculation of the arithmetic mean of the seven samples taken during treatment.

Hemoglobin and methemoglobin. Hemoglobin and methemoglobin determinations were performed daily on venous blood during treatment using the method of Wendell (17).

⁴ SN-8,233, SN-11,191, and SN-11,226 were tested at the maximum tolerated dose as estimated from monkey toxicity. SN-1,452 was tested at half the estimated maximum tolerated dose. Agranulocytosis (SN-8,233), granulocytopenia (SN-11,191 and SN-11,226), drug fever (SN-1,452) and high methemoglobinemia (SN-8,233 and SN-11,191) were encountered. One of three subjects to whom SN-8,233 was administered died of myocardial infarction nine days after drug was stopped. Death was thought to be unrelated to drug toxicity (1). Incidentally, no unequivocal therapeutic effect was obtained.

TABLE II

Therapeutic effect in sporozoite-induced vivax malaria of eighteen 8-aminoquinolines administered for 14 days concurrently with quinine

Drug	Daily dose (base)	Number of subjects	Mean concentration of drug in plasma				Relapse ratio Individuals relapsed Individuals treated		Duration of subsequent parasitic latent period in subjects who relapsed		Duration of follow-up period in subjects who did not relapse	
			Test drug		Quinine		Moderate* infections	Severe* infections	Moderate* infections	Severe* infections	Moderate* infections	Severe* infections
			Range	Mean	Range	Mean						
SN-191	mgm. 64	5	gamma per liter no method	gamma per liter —	mgm. per liter 5-6	5	1/1	4/4	days 13	days 9, 11, 18, 13	days	days
SN-9,972	7.5	5	no method	—	7-8	7	—	3/5		12, 16, 62		124, 137
	15	12	no method	—	5-10	8	0/2	2/10		78, 11	214, 164	122, 118, 114, 117, 118, 112, 81, 81
SN-11,191	30	5	92-232	143	6-11	9	—	5/5		29, 37, 26, 11, 16		
SN-12,325	30	5	80-103	94	5-10	8	0/3	2/2		14, 12	304, 301, 296	
	60	5	166-307	247	6-11	9	2/3	1/2	52, 33	106	216	213
SN-12,352	30	5	0-25	12	6-10	8	1/2	3/3	12	10, 8, 20	268	
	60	5	27-43	35	6-9	7	2/3	1/2	12, 44	19	227	227
SN-12,354	30	2	6-9	8	7-10	8	—	2/2		18, 35		
	60	5	10-31	15	7-9	8	1/3	2/2	26	14, 10	328, 262	
SN-12,451	30	3	59-84	75	9-10	10	0/1	2/2		28, 10	281	
	60	5	83-216	147	6-10	8	0/1	3/4		12, 49, 12	245	250
SN-13,232	30	5	76-123	92	5-11	8	0/2	3/3		28, 8, 13	352, 302	
	60	5	185-291	211	8-11	9	1/2	3/3	53	19, 23, 49	236	
SN-13,233	60	5	65-156	114	8-11	9	0/1	3/4		16, 10, 10	266	280
SN-13,274	30	5	24-41	32	5-11	8	1/1	2/4	15	13, 19		222, 222
	60	10	26-285	70	6-9	7	0/5	1/5		23	157, 167, 158, 152, 107	83, 83, 83, 80
SN-13,276	15	5	30-51	40	7-11	9	—	5/5		21, 15, 37, 12, 9		
	30	10	33-67	44	5-10	8	0/1	8/9		41, 41, 17, 48, 11, 38, 64, 12	187	192
	45	5	24-58	43	5-10	8	0/3	2/2		15, 14	208, 183, 181	
	60	43	26-196	63	3-11	7	1/26	3/17	22	70, 45, 54	188, 179, 177, 166, 164, 165, 164, 143, 158, 149, 131, 130, 124, 149, 122, 116, 111, 108, 105, 76, 73, 73, 65, 63, 80	370, 380, 352, 349, 327, 271, 270, 270, 267, 124, 81, 81, 61, 65

TABLE II—Continued

Drug	Daily dose (base)	Number of subjects	Mean concentration of drug in plasma				Relapse ratio Individuals relapsed Individuals treated		Duration of subsequent parasitic latent period in subjects who relapsed		Duration of follow-up period in subjects who did not relapse	
			Test drug		Quinine		Moderate* infections	Severe* infections	Moderate* infections	Severe* infections	Moderate* infections	Severe* infections
			Range	Mean	Range	Mean						
SN-13,380	mgm. 60	5	gamma per liter 99-251	gamma per liter 152	mgm. per liter 6-12	mgm. per liter 9	1/2	3/3	days 78	days 16, 13, 12	days 282	days
SN-13,429	30	5	22-37	32	7-10	9	0/1	4/4		39, 11, 40, 10	276	
	60	5	37-128	51	8-10	9	—	2/5		104, 63		120, 120, 120
SN-13,619	60	5	197-328	242	8-10	7	—	5/5		16, 9, 31, 13, 10		
SN-13,694	30	5	no method	—	6-11	8	1/1	4/4	31	9, 28, 11, 12		
	60	5	no method	—	6-11	8	—	4/5		24, 12, 10, 10		126
SN-13,697	120	5	no method	—	7-10	7	1/1	4/4	11	8, 11, 16, 10		
SN-14,011	3.7	5	no method	—	6-10	8	—	5/5		8, 11, 9, 8, 9		
	7.5	5	no method	—	5-10	8	—	5/5		7, 9, 10, 10, 9		
	15	5	no method	—	5-9	8	—	5/5		8, 8, 10, 8, 14		
	30	5	no method	—	5-10	8	—	5/5		12, 12, 10, 9, 23		
DR-15,302	30	5	no method	—	5-9	7	—	2/5		16, 15		96, 89, 84
	60	5	no method	—	5-10	8	—	2/5		74, 76		93, 91, 91

* Infections were classified as "severe" when the prepatent period or preceding latent interval was less than 15 days or 30 days, respectively. When these periods were longer, the infections were classified as "moderate."

RESULTS

Antimalarial activity

Table II summarizes the data on the antimalarial activity of 18 compounds that we have studied systematically. Thirty-four drug regimes in 65 moderate and 165 severe therapeutic tests are tabulated.

Relapse rate. Eight of the drugs were ineffective at the dosages studied in clinical attacks that represented severe therapeutic tests. All the subjects treated with these drugs subsequently underwent relapse.

Ten compounds apparently cured one or more

patients whose infection constituted a severe therapeutic challenge. Five of these drugs showed pronounced activity in that the majority of subjects treated with them have not subsequently relapsed. Follow-up observations on these patients have been made from two months to one year. This group of drugs consists of SN-9,972, SN-13,274, SN-13,429, and DR-15,302. SN-13,276 (pentaquine) has cured 14 out of 17 infections that fall into the severe test group. Studies on this drug have been expanded to include curative trials at different dosages with and without concurrent quinine, prophylactic tests, and more detailed studies on its toxicology in man (18, 19).

Parasite clearance. The eighteen 8-aminoquinolines as a group, administered with quinine, terminated individual attacks no more rapidly than quinine alone. The 34 regimes were divided into two groups; the first consisted of those on which all relapsed, the second included those on which one or more individuals failed to relapse. The time required for clearance of parasites from the peripheral blood was then studied for all patients falling into each of the two groups. The difference between the mean parasite clearance times of the two groups was not statistically significant.

Latent period. The cumulative frequency of latent intervals in 110 subjects who relapsed after treatment with these drugs is shown in Figure 2. All subjects had infections classified as severe therapeutic tests. Fifty per cent of the cases relapsed within two weeks and 90 per cent within six weeks. These figures lend additional significance to the length of the follow-up periods noted in Table II.

The latent interval ogive may be broken down into three separate curves. The 34 regimes were divided into three groups according to the relapse ratio for therapeutic tests. The first group consisted of 73 attacks on 20 drug regimes on which all patients underwent subsequent relapse. The second group was comprised of 21 patients who relapsed on five drug regimes which produced a relapse rate of below 100 per cent but above 50

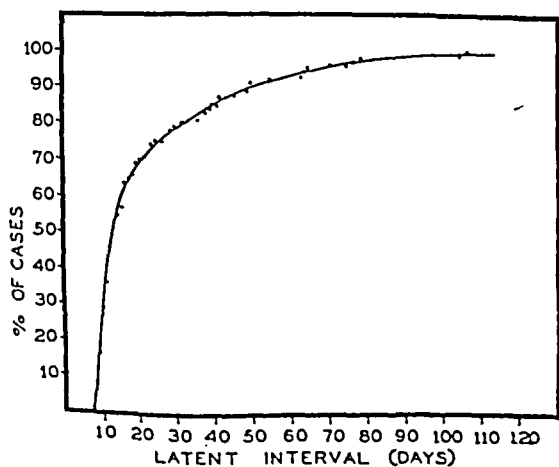


FIG. 2. CUMULATIVE FREQUENCY OF LENGTH OF LATENT INTERVALS IN 110 PATIENTS WHO RELAPSED AFTER TREATMENT WITH 8-AMINOQUINOLINES, ADMINISTERED CONCURRENTLY WITH QUININE

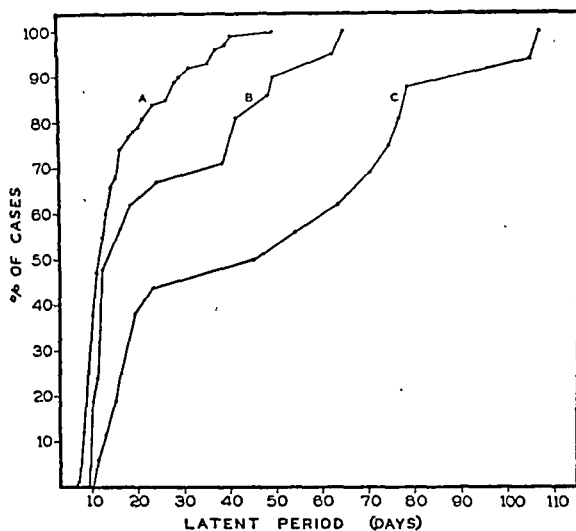


FIG. 3. BREAKDOWN OF CUMULATIVE FREQUENCY OGIVE SHOWN IN FIGURE 2

Curve A is based on the latent intervals of 73 patients on 20 drug regimes after which all patients relapsed. Curve B is based on the latent intervals of 21 patients on five drug regimes after each of which more than one-half of the patients relapsed. Curve C is based on latent intervals of 16 patients on seven drug regimes after each of which half or less than half of the patients relapsed.

per cent. The third group was composed of 16 patients who relapsed on seven drug regimes on which 50 per cent or less of the patients relapsed. The curves are shown in Figure 3. It is apparent that drug regimes which produce a high percentage of cures, tend to prolong the subsequent latent intervals in patients whom they fail to cure.

Plasma concentrations. The plasma concentrations of the 8-aminoquinolines varied widely. In general, there was a very low positive correlation between oral dose and plasma concentrations but there were large individual variations. The concentration of the 8-aminoquinolines in the plasma could be correlated neither with the subsequent latent interval in those individuals who relapsed nor with relapse or failure to relapse. However, the total number of patients on any one drug regime was low.

The quinine plasma concentrations ranged between 3 mgm. per liter and 16 mgm. per liter in an approximately Gaussian distribution, with the mode occurring in the region of 8 mgm. per liter. There was no relation between the mean plasma quinine concentration attained during treatment

and the length of the latent interval in those individuals who relapsed, nor was there any relation between quinine level and occurrence of relapse or failure to relapse.

When treatment was discontinued, the 8-aminoquinolines and quinine both disappeared rapidly from the plasma.

TOXICITY

Table III summarizes the data on the toxicity of the eighteen 8-aminoquinolines when given over a 14-day period concurrently with quinine.

Symptomatology

The symptoms produced by these regimes were qualitatively similar to those caused by pamaquin (9). At 15 mgm. of pamaquin daily, there were practically no symptoms, while at 63 mgm., abdominal pain, anorexia, nausea and vomiting were frequently severe and in several cases necessitated discontinuance of the medication before the 14 days of drug administration were completed. In Table III are indicated the clinical toxicities of each of the drug regimes tested and, as well, the

TABLE III

Toxicity in man of a group of eighteen 8-aminoquinolines administered for 14 days concurrently with quinine

Drug	Daily dose (base)	Number of patients	Number of patients showing commonly observed symptoms			Number of patients showing leucocytosis of 12,000 or over	Average methemoglobinemia* on last 4 days of treatment per cent of total hemoglobin		Approximate pamaquin (base) equivalent (concurrent with quinine)	Comment
			Mild	Moderate	Severe		Range	Mean		
SN-191	mgm. 64	5	1	3	0	2	1.4-1.8	1.6	mgm. per day 30	1 patient had temperature of 100.6 on 10th day.
SN-9,972	7½	5	1	1	0	1	1.8-3.1	2.2	15	
	15	12	3	4	0	4	1.9-5.9	3.9	30	1 patient had white count of 3,700 on 14th day.
SN-11,191	30	5	2	1	0	0	3.1-4.5	3.7	15-30	
SN-12,325	30	5	2	1	0	3	2.7-4.6	3.5	15	
	60	5	1	3	0	0	4.1-7.6	5.6	45	1 patient showed white count of 3,000-4,000 toward end of course
SN-12,352	30	5	1	0	0	1	1.8-4.1	2.9	15	
	60	5	3	3	0	1	1.2-4.5	2.8	15	
SN-12,354	30	2	0	0	0	0	2.8-3.1	2.9	15	
	60	5	1	2	0	3	2.7-6.2	4.6	30	
SN-12,451	30	3	1	0	0	1	3.0-5.9	4.1	15-30	
	60	5	2	1	0	2	3.2-7.2	4.9	30	
SN-13,232	30	5	0	1	0	2	2.5-4.7	3.6	15	
	60	5	0	1	1	2	2.7-4.6	3.0	15	Pruritic vesicular rash in 2 patients at start of course, disappeared in 5 days. Fever of 101.2 on 8th day in 1 patient.
SN-13,233	60	5	2	1	2	1	2.9-6.4	4.8	30	Fever of 100.4-100.8 on 8th and 9th days in 1 patient.
SN-13,274	30	5	0	4	1	1	2.3-3.0	2.4	30	
	60	10	5	4	0	8	2.1-7.6	4.8	30	

TABLE III—Continued

Drug	Daily dose (base)	Number of patients	Number of patients showing commonly observed symptoms			Number of patients showing leucocytosis of 12,000 or over	Average methemoglobinemia* on last 4 days of treatment per cent of total hemoglobin		Approximate pamaquin (base) equivalent (concurrent with quinine)	Comment
			Mild	Moderate	Severe		Range	Mean		
SN-13,276	mgm. 15	5	2	0	0	1	2.1-2.5	2.3	mgm. per day 15	1 patient had a macular erythematous rash.
	30	10	4	1	0	2	1.0-4.9	2.8	15	
	45	5	2	1	0	0	2.1-2.8	2.6	15	
	60	43	11	19	1	8	0.8-10.2	3.7	30	2 individuals had fever; one of 102 on 8th day and the other of 100.6 and 101.4 on the 5th and 6th days.
SN-13,380	60	5	1	0	3	0	2.4-4.2	3.1	15	
SN-13,429	30	5	3	2	0	1	1.7-3.2	2.5	15	
	60	5	2	1	0	3	2.2-8.3	4.5	30	
SN-13,619	60	5	2	1	0	2	1.1-1.7	1.5	15	1 patient complained of pruritis of palm and soles during entire course of therapy. No objective changes.
SN-13,694	30	5	1	2	0	3	1.1-1.4	1.3	15	
	60	5	1	0	0	1	0.7-1.6	1.1	0	
SN-13,697	120	5	1	2	0	2	1.4-2.1	1.5	15	
SN-14,011	3.7	5	1	0	0	2	1.3-2.3	1.6	0	
	7.5	5	1	0	0	1	1.2-1.5	1.3	0	Temperature of 100.4 on 8th day in one patient.
	15	5	0	0	0	1	1.3-2.4	1.5	0	
	30	5	0	0	0	3	2.0-3.1	2.7	0	
DR-15,302	30	5	1	1	0	2	3.0-3.9	3.5	15	
	60	5	0	1	0	1	4.1-6.8	5.4	30	

* By the method used (17) the mean methemoglobin concentration of 187 normal individuals was 1.8 per cent of the total hemoglobin (standard deviation: 1.0).

approximate daily dosage equivalent of pamaquin (base).

The most common symptom was pain. Frequently after the first two or three days the patients noted epigastric discomfort which seemed to spread into the retrosternal area, and in a few instances to the shoulder, to the back, neck, or inguinal region. The ache or pain was constant with periodic exacerbation, accompanied in most cases by epigastric tenderness. Analgesics were

usually withheld in order not to mask the symptoms of toxicity.

In some instances the pain diminished or disappeared after the first three to eight days of drug administration, but in many it persisted throughout treatment, subsiding gradually within the next two or three days. Other symptoms, all transient, occurring with less uniformity in the various groups, included anorexia, nausea and vomiting, headache, weakness, and diarrhea.

Physical observations

Except for cyanosis, which was observed when methemoglobinemia exceeded 5 or 6 per cent, few abnormalities were found on physical examination. Slight pallor was sometimes seen, and epigastric tenderness was common.

Six patients had unexplained fever of 100° to 102° F. rectally on the fifth to tenth day of medication, the rise most commonly occurring on the eighth day. The fever rapidly subsided in all cases although treatment was not interrupted. Two of the six patients received SN-13,276 at 60 mgm. per day; the other four patients were on other drug regimes.

Skin eruptions occurred in three patients. They were mild and transient. Two were vesicular and pruritic, the other was macular and erythematous. All disappeared after treatment was discontinued.

Laboratory observations

Leucocytes. Leucopenia between 3,000 and 4,000 per cu. mm. was observed in two patients (SN-9,972 and SN-12,325). In both, the low counts were recorded at the end of therapy and rapidly returned to normal.

Low-grade leucocytosis was observed in the last three to seven days of the 14-day course of treatment in 66 individuals. The white counts usually rose to 12,000 to 14,000 per cu. mm. but in an occasional subject attained somewhat higher values. The leucocytosis was not associated with the drug fever mentioned above.

Hemoglobin. Except in the case of pentaquine (SN-13,276) the number of patients on any individual regime was too small for statistical evaluation of change in hemoglobin. When SN-13,276 at 60 mgm. of base per day was administered with quinine there occurred a mean fall of 1 gram of hemoglobin (18). If, however, all other regimes are considered together as one group no net decrease in hemoglobin can be demonstrated.

Methemoglobinemia. Methemoglobinemia was produced by all regimes studied except those with SN-191, SN-13,619, SN-13,694, SN-13,697 and lower doses of SN-14,011.

In a given regime the amount of methemoglobinemia varied considerably from individual to individual but not from day to day in the same

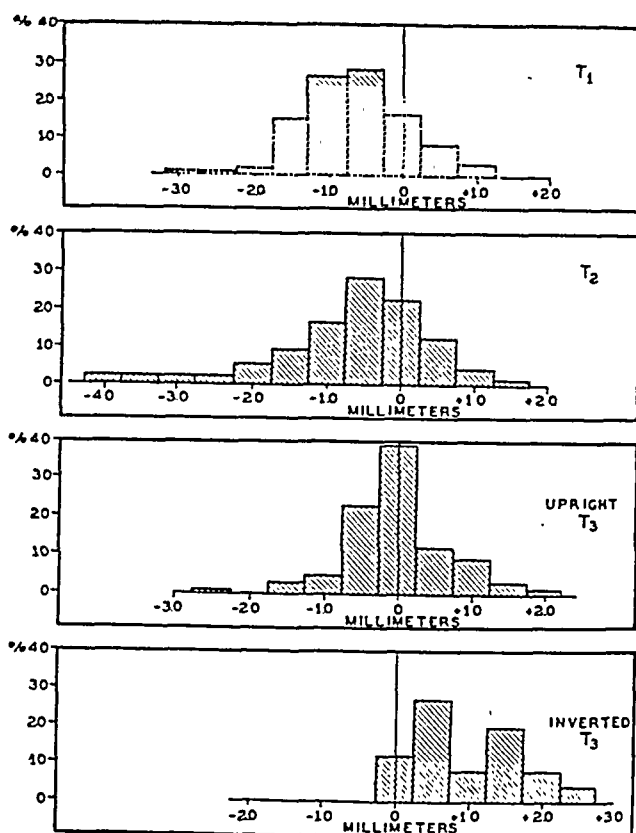


FIG. 4. HISTOGRAMS OF CHANGES IN THE HEIGHT OF T WAVES AFTER THE CONCURRENT ADMINISTRATION OF 8-AMINOQUINOLINES AND QUININE TO 187 PATIENTS DURING ACUTE ATTACKS OF VIVAX MALARIA

individual. The average of the mean methemoglobinemia during the last four days of treatment for all individuals, and the range of the four-day mean for each individual are shown in Table III.

With the method employed to determine methemoglobin (17) the mean value obtained in the study of 187 normal individuals was 1.8 per cent of the total hemoglobin. (Standard deviation = 1.0.)

Electrocardiographic changes. The electrocardiograms of 187 individuals who had recently completed a 14-day course of an 8-aminoquinoline plus quinine, were studied. Clinical malaria was present only during the first few days of the course of drug administration. The only statistically significant changes noted were diminution of the amplitude of T₁ and of T₂, and of T₃ when initially upright. When T₃ was initially inverted, it became shallower, or, on occasion, became upright. The distribution and magnitudes of these changes are shown in Figure 4. Previously upright T waves occasionally became inverted in Lead III. This occurred in only one instance in Lead II

(SN-14,011 + quinine) but never in Lead I. The drug regimes accounting for the greatest T wave changes included the following four drugs (all administered with quinine): SN-9,972, SN-13,274, SN-13,276, and SN-14,011. In instances where follow-up electrocardiograms were taken, the T-waves rapidly returned to their initial amplitude. We have observed similar changes in drug regimes consisting of 8-aminoquinolines administered without quinine.

DISCUSSION

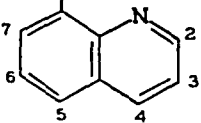
Although the toxicity of 8-aminoquinolines as revealed by animal experiments can serve only as

a rough guide to human toxicity, it has been found practical and relatively safe in the study of drugs herein reported to translate the toxicity in the monkey to man, beginning human tests at a dosage estimated to be equivalent to one-sixth the maximum tolerated dose on the basis of comparison with pamaquin. No serious toxicity has been encountered in clinical tests on these regimes. The most prominent symptoms have been abdominal pain, anorexia, nausea and vomiting.

Cyanosis has occurred when methemoglobinemia exceeded 5 or 6 per cent of the total hemoglobin. Mild drug fever, leukocytosis and leukopenia have been observed. The only 8-aminoquinolines that

TABLE IV

Data on ten 8-aminoquinolines which have "cured" one or more infections presenting a severe therapeutic challenge

Survey number	General formula of 8-(ω -aminoalkylamino) quinolines				Daily dosage	Toxicity: approximate pamaquin daily dosage equivalent (concurrent with quinine)	Relapse ratio: $\frac{\text{Individuals relapsed}}{\text{Individuals treated}}$	
	$R'-N-R''$	$-(CH_2)_n-$	Nuclear substituents				Moderate* infections	Severe* infections
							mgm.	mgm. per day
Drugs which have "cured" one infection presenting severe challenge								
SN-12,352	amino	n-hexyl	6-methoxy	60	15	2/3	1/2	
SN-12,451	ethylamino			60	30	0/1	3/4	
SN-13,233	n-propylamino			60	30	0/1	3/4	
SN-12,325	methyl isopropylamino			60	45	2/3	1/2	
SN-13,694	diethylamino		5-chloro; 6-methoxy	60	0	—	4/5	
Drugs which have "cured" more than one infection presenting a severe challenge								
SN-13,274	isopropylamino	1-methylbutyl	6-methoxy	60	30	0/5	1/5	
SN-9,972			5,6-dimethoxy	15	30	0/2	2/10	
SN-13,276		n-amyl	6-methoxy	60	30	1/26	3/17	
DR-15,302			4-methyl; 6-methoxy	60	30	—	2/5	
SN-13,429	n-propylamino	1-methylbutyl	6-methoxy	60	30	—	2/5	

* Infections were classified as "severe" when the prepatent period or preceding latent interval was less than 15 days or 30 days, respectively. When these periods were longer, the infections were classified as "moderate."

have not caused significant methemoglobinemia at any dosage level studied are SN-191, SN-13,619, SN-13,694 and SN-13,697. Three of these (SN-191, SN-13,619, and SN-13,697) have no 6-methoxy radical on the nucleus. The fourth has a chloro substituent in the 5 position as well as a 6-methoxy. The T wave changes that have been noted are probably non-specific and of dubious significance because they were not attended by other evidence of cardiovascular abnormality and similar changes have been reported with divers other drugs (20 to 25).

These studies indicate that the curative properties of pamaquin are not unique to that drug but are shared by several analogues. Nevertheless, the present data do not warrant definite conclusions as to the specific molecular configuration required in a curative drug. Certain tentative inferences, however, suggest themselves.

SN-9,972, SN-13,274, SN-13,276, SN-13,429 and DR-15,302 apparently cured two or more patients (Table IV) when administered concurrently with quinine. They are all secondary amines. The alkyl substituent of the terminal amine nitrogen is isopropyl in four of these drugs and normal propyl in the fifth. All five compounds possess five carbons in the inter-nitrogen aliphatic chain; in three of them it is a secondary amyl and in two it is normal amyl (Table IV). All have a 6-methoxy group on the quinoline nucleus. On an equal weight basis these drugs have greater therapeutic efficacy than pamaquin. SN-13,276 (pentaquine) has been the most extensively studied of these drugs. Investigations on pentaquine are reported in detail elsewhere (18, 19).

An additional methoxy group in the 5 position on the quinoline ring appeared to increase the toxicity of the drug. Thus, SN-9,972 is the 5,6-dimethoxy homologue of SN-13,274, and SN-12,354 is the dimethoxy homologue of SN-12,352. In each case, the monomethoxy compound produced less toxicity than the dimethoxy derivative. This is further borne out in the case of SN-8,233,⁴ which is the 5,6-dimethoxy homologue of pamaquin.

The effectiveness of drugs as curative agents was demonstrated not only by their immediate effect on the relapse ratio but also by their effect on subsequent latent intervals. Drug regimes which produced a high percentage of cures, tended to pro-

long the subsequent latent intervals of patients whom they failed to cure. This prolongation was more pronounced with drug regimes which produced a marked lowering of the relapse ratio than with those that decreased the relapse ratio only slightly. The lengthening of the latent interval implies that the disease has been modified and that the relapse rate after subsequent therapy will be lower. The effect of these drugs, therefore, on the clinical course of malaria is greater than is apparent from a study of the immediate relapse ratio alone.

SUMMARY

1. Eighteen 8-aminoquinolines have been studied for antimalarial activity in human *vivax* malaria (Chesson strain) and for their clinical toxicity.
2. The relapse rate in this disease is lowered by SN-9,972, SN-13,274, SN-13,276, SN-13,429 and DR-15,302.
3. SN-13,276 (pentaquine) has been the most extensively studied drug of this group. On an equal weight basis it has greater curative activity and lower toxicity than pamaquin (plasmochin).
4. Drug regimes which produced a high percentage of cures tended to prolong the subsequent latent intervals in patients whom they failed to cure.

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COMPARISON OF CHLOROQUINE, QUINACRINE (ATABRINE), AND QUININE IN THE TREATMENT OF ACUTE ATTACKS OF SPOROZOITE-INDUCED *VIVAX* MALARIA (CHESSON STRAIN)¹

PRELIMINARY REPORT

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Chloroquine, 7-chloro-4-(4-diethylamino-1-methyl-butylamino)-quinoline, a synthetic anti-malarial agent developed during the war, has proved superior to atabrine and quinine (1). The following investigations were designed to supplement other observations in induced malaria and field observations by a study of the relative effectiveness of chloroquine, quinacrine, and quinine in the treatment of acute attacks of standardized sporozoite-induced *vivax* infections.

PROCEDURES AND METHODS

General

Details of the general procedure and the plan of investigation are reported elsewhere (2). Thirty-nine healthy Caucasian volunteers at Stateville Penitentiary were infected with South Pacific *vivax* malaria (Chesson strain) by the bites of ten infected *Anopheles quadrimaculatus* mosquitoes or by injection of their infected salivary glands.³ The Chesson strain (3) was chosen

¹ This investigation was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The work was planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for Coordination of Malarial Studies. This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General, U. S. Army.

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² Captain, M.C., A.U.S.

³ The mosquitoes used in these studies were provided by Dr. Clay G. Huff and Dr. Frederick A. Coulston of

because it is characterized by a high relapse rate, short latent interval between relapses, and almost complete absence of delayed primary attacks (4, 5, 6). Evaluation of results could be made with a small number of subjects because the complete history of each infection was known; and because the effect of immunity on relapse rate was minimized by utilizing presumably susceptible individuals living in a non-endemic area, and by limiting therapeutic trials to primary attacks and early relapses. In addition, treatment was initiated early in most of the attacks.⁴

Drug administration

Chloroquine. A total of 2 grams of base (equivalent to 3.2 grams of the diphosphate) was administered over a period of one week. After an initial dose of 0.4 gram of base, followed by two doses of 0.2 gram at four-hour intervals, the daily maintenance was 0.2 gram.

Quinacrine. After an initial dose of 0.4 gram of quinacrine dihydrochloride (approximately 80 per cent base) followed by three of 0.2 gram at four-hour intervals, the daily maintenance dose was 0.4 gram. The total amount of quinacrine for seven days was 3.4 grams of the salt.

Quinine. Two groups of subjects received quinine; one for seven days and one for 14 days. In the first group, a total of 11-12 grams of base (approximately equivalent to 13-15 grams of the hydrochloride or sulfate) was administered over seven days. In the second group, a total of 21-23 grams of base was administered over a 14-day period.

Analysis of drugs in plasma

Whole blood was centrifuged for 15 minutes at 2,000 r.p.m.; the plasma was separated and recentrifuged for

the Department of Bacteriology and Parasitology. They supervised infection of mosquitoes, inoculation of volunteers and determined the intensity of infection in the salivary glands of the mosquitoes.

⁴ The investigations reported in this paper would not have been possible except for the enthusiastic cooperation of the inmates and the administrative staff of Stateville Penitentiary.

an hour at the same speed in order to insure complete removal of the components of the buffy coat. The anticoagulant used was potassium oxalate. Quinine and quinacrine were estimated in the plasma by the methods of Brodie and Udenfriend (7, 8). Chloroquine was estimated by a modification (9) of the method of Brodie, *et al.* (10).

RESULTS

Time necessary for the clearance of parasites during treatment. Chloroquine cleared the blood of parasites more rapidly than quinacrine, which in turn required less time than quinine to produce negative thick smears of the peripheral blood. Three-quarters of the patients treated with chloroquine had negative thick smears two days after therapy had been instituted; with quinacrine, three days were necessary, and with quinine, four days (Figure 1). Patients treated with chloroquine on the average became afebrile sooner than those receiving the other drugs.

Relapse rate. The Chesson strain, studied under these conditions, has a higher relapse rate (5) than that usually seen in naturally acquired Southwest Pacific *vivax* malaria in non-immune military personnel during World War II (11). In these studies the rate was approximately the same with each drug (Figure 2 and Tables I, II, III, IV). Two patients did not relapse after treatment with quinine. One of the patients had a markedly prolonged prepatent period (57 days) and the other a long parasitic latent period (39 days) following treatment of the preceding attack. Analysis of data from over one hundred clinical attacks studied under these conditions has revealed that pa-

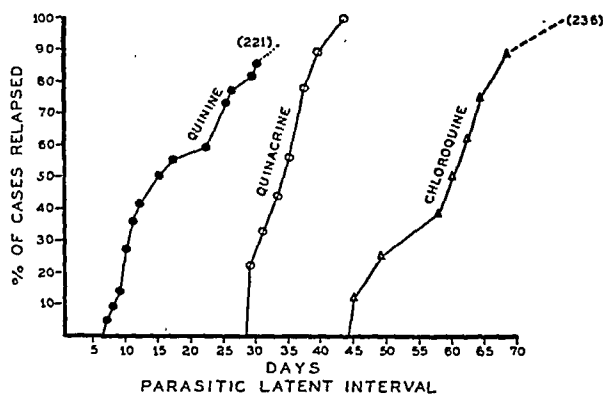


FIG. 2

tients in whom the prepatent period exceeds 14 days or the preceding latent period 30 days have much lower relapse rates than do patients in whom these periods are shorter (5).

Parasitic latent period after termination of treatment. The median length of the period of parasitic latency following termination of therapy with chloroquine was almost twice as great as that following quinacrine and four times as great as that after quinine (Figure 2, Tables I, II, III, IV). In 50 per cent of the patients treated with quinine, parasitemia recurred in 15 days or less. With quinacrine, 50 per cent relapsed within 34 days, whereas, with chloroquine, 60 days elapsed before parasitemia reappeared in one-half the group.

One of the patients treated with quinine for 14 days did not undergo parasitic relapse until 221 days, and one of the patients treated with chloroquine did not relapse until 236 days. The former subject had a latent period of 38 days after his primary attack and probably presented a less severe challenge to the drug. The latter patient was permitted to undergo an unusually long febrile course both during the primary attack and first relapse, and, therefore, acquired immunity may have been a factor in prolonging his latent period after treatment with chloroquine.

Drug dosage and concentration of drug in the plasma. The dosages of drugs used in these studies were probably as high as can be given in large scale treatment with reasonable expectation of avoiding severe toxicity. With quinine, in fact, moderate symptoms of cinchonism were encountered.

The average of the mean plasma drug levels of

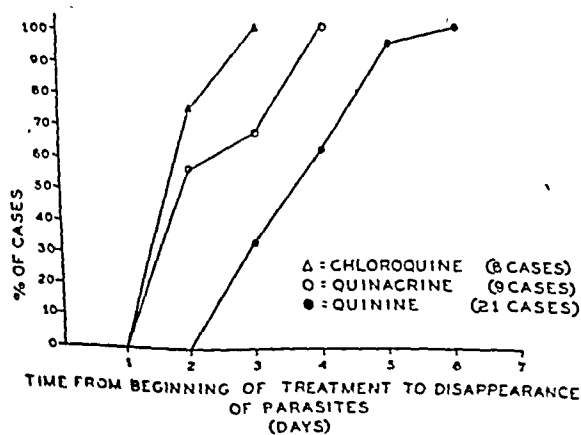


FIG. 1

TABLE I

Therapeutic effect of quinine, administered for seven days

Patient	Nature of attack*	Range of plasma concentration	7-day mean plasma concentration	Parasite clearance	Interval from end of therapy to relapse	
					Fever	Parasites
		mgm./L.	mgm./L.	days	days	days
1	P	5.9-18	9.7	5	8	7
2	P	4.0-16	9.4	5	12	10
3	P	8.8-27	16	4	19	15
4	P _v	3.9-11	8.1	4	10	10
5	P _v	3.3-16	8.4	6	12	9
6	P _v ¹²	5.1-19	12	5	15	11
7	P _e ¹¹	6.8-15	10	5	14	11

* P indicates a primary attack. The numerical exponents indicate the number of days of fever and/or parasitemia experienced by certain patients treated late during the course of an attack. The subscripts _v and _e indicate, respectively, intravenous or intracutaneous inoculations of dissected mosquito salivary glands.

all patients receiving quinine (8 mgm. per liter) was about $1\frac{1}{3}$ times the concentration necessary to obtain a therapeutic effect (12). For the group receiving quinacrine the average plasma concentration (71 gamma per liter) was slightly greater than twice the minimum effective therapeutic level (12). In the group receiving chloroquine, however, the mean plasma concentration attained (96 gamma per liter) was at least six times the minimum required to eradicate the blood forms of the parasite (13).

TABLE II

Therapeutic effect of quinine, administered for 14 days

Patient	Nature of attack*	Range of plasma concentration	14-day mean plasma concentration	Parasite clearance	Interval from end of therapy to relapse	
					Fever	Parasites
		mgm./L.	mgm./L.	days	days	days
1	P	5.1-16	7.9	4	13	10
2	P	5.0-13	7.3	—	—	—
3	P	6.9-21	10	5	12	8
4	R ₁	5.3-16	6.6	5	16	12
5	R ₁	0.5-3.6	2.6	5	228	221
6	R ₂	4.1-11	6.0	4	32	25
7	R ₃	5.2-9.6	6.8	3	21	15
8	R ₃	4.0-8.8	6.0	3	—	25
9	R ₃	4.0-9.6	5.4	3	—	22
10	R ₃	3.4-5.9	4.9	3	20	17
11	R ₃	8.4-9.4	8.8	3	34	29
12	R ₃	4.6-7.8	6.4	4	34	26
13	R ₃	6.9-11	7.6	3	35	30
14	R ₃	7.0-13	10	3	30	25
15	R ₃	5.6-8.5	7.1	4	—	—

* P indicates a primary attack. R₁, R₂, and R₃ indicate first, second and third relapses.

DISCUSSION

The two groups of patients treated with quinine differed significantly from each other in mean parasite clearance time and mean parasitic latent interval after therapy. The seven-day group showed a longer clearance time and a shorter latent interval than the 14-day group. The seven-day group consisted entirely of primary attacks whereas the 14-day group contained a large percentage of third relapses. The attacks treated with chloroquine were primaries and first and second relapses. They were probably intermediary in severity of infection between the two qui-

TABLE III

Therapeutic effect of quinacrine, administered for seven days

Patient	Nature of attack*	Range of plasma concentration	7-day mean plasma concentration	Parasite clearance	Interval from end of therapy to relapse	
					Fever	Parasites
		gamma/L.	gamma/L.	days	days	days
1	P	40-90	61	3	34	29
2	P	92-105	151	2	44	39
3	P ₁ ¹⁹	43-77	65	4	39	37
4	P ₁ ²¹	52-80	59	2	—	43
5	P _v	39-95	62	2	29	29
6	P _v	46-70	54	2	—	35
7	P _v	70-117	83	4	—	33
8	P ₂ ²²	35-61	43	4	37	31
9	R ₁	50-79	62	2	39	37

* P indicates a primary attack. R₁ indicates first relapse. The numerical exponents indicate the number of days of fever and/or parasitemia experienced by certain patients treated late in the course of an attack. The subscripts _v and ₁ indicate, respectively, intravenous or lymph gland inoculations of dissected mosquito salivary glands.

nine groups. Furthermore, the difference in means between the quinine groups were smaller than the differences in means of all the quinine-treated patients and those treated with quinacrine and chloroquine. For these reasons, the two quinine series were considered as one group for purposes of comparison.

In the series here reported, chloroquine cleared the peripheral blood more rapidly in the majority of cases, than quinacrine and quinine. These results are consonant with findings obtained elsewhere in a large group of naturally occurring attacks (15). However, due to the small number of cases and to the fact that parasite clearance time was measured in days rather than in hours,

TABLE IV

Therapeutic effect of chloroquine, administered for seven days

Patient	Nature of attack*	Range of plasma concentration	7-day mean plasma concentration	Parasite clearance	Interval from end of therapy to relapse	
					Fever	Parasites
		gamma/L.	gamma/L.	days	days	days
1	R ₁	57-100	77	2	50	45
2	R ₁	69-103	83	2	52	49
3	R ₁	100-164	129	2	70	64
4	P _o	62-103	85	3	63	58
5	R ₂	58-88	74	2	—	60
6	P	95-152	125	3	71	68
7	R ₂	70-130	110	2	—	62
8	R ₁ ²⁶	73-110	89	2	241	236

* P indicates a primary attack. R₁ and R₂ indicate first and second relapses. The numerical exponent indicates the number of days of parasitemia experienced by one patient treated late in the course of an attack. The subscript o indicates subcutaneous inoculation of dissected mosquito salivary glands.

the difference between the mean parasite clearance time here reported between chloroquine and quinacrine, could not be proved statistically significant. On the other hand, the differences in mean clearance time observed between quinine and quinacrine and between quinine and chloroquine were sufficiently great for the size and dispersion of the series, for them to be considered due to factors other than chance.

The parasitic latent interval following treatment with chloroquine was longer than that following quinacrine and quinine. Furthermore, the margin between the minimal plasma concentration of chloroquine required to eradicate trophozoites and the concentration that may be attained without encountering toxicity is much greater than are the margins for quinacrine and quinine.

The long period of latency following treatment with chloroquine may be attributed, in part, to its slow rates of excretion and degradation; but the long persistence of an effective suppressive plasma concentration is also due to the fact that many times the minimal effective plasma concentration may safely be attained during therapy.

The results that we have obtained using experimental sporozoite-induced infection are in accord with previous studies in artificially induced infection (4, 13, 14) and with concurrent studies in naturally acquired malaria (15).

SUMMARY

1. Chloroquine has been tested in a small series of infections with highly relapsing Chesson strain of Southwest Pacific *vivax* malaria, under controlled conditions and compared with quinine and quinacrine.

2. Both chloroquine and quinacrine cleared the blood of parasites in most of the cases more rapidly than did quinine.

3. The relapse rate after treatment with all three drugs was about the same, 90 per cent or over.

4. The latent period following therapy for 50 per cent of patients treated with quinine, quinacrine, and chloroquine, was 15 days, 34 days, and 64 days, respectively.

5. Chloroquine was superior to quinacrine and quinine for the treatment of this series of acute attacks of *vivax* malaria.

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THE THERAPEUTIC EFFECTIVENESS OF LARGE DOSES OF PALUDRINE IN ACUTE ATTACKS OF SPOROZOITE-INDUCED *VIVAX* MALARIA (CHESSON STRAIN)¹

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INTRODUCTION

Paludrine is a new antimalarial agent which was developed in England during World War II. In an extensive investigation of the antimalarial activity of pyrimidine derivatives, Curd, Davey, and Rose (1, 2) synthesized the drug, N₁-p-chlorophenyl-N₈-isopropylbiguanide, in 1945 and demonstrated that it exhibited a high degree of activity in avian malaria. Maegraith, Adams and their co-workers (3, 4, 5) tested this compound in human infections and found that it was a highly effective agent in the treatment of both *vivax* and *falciparum* malaria in man. Paludrine has been studied extensively by English (5) and Australian investigators (6) and its activity and usefulness have been confirmed. Maegraith *et al.* (5) have reported that a single dose of 50 to 400 mgm. will

produce clinical cure of relapses and delayed primary attacks of naturally acquired *vivax* malaria. The drug is well tolerated in doses as high as 1.5 grams a day for 14 to 28 days. In 157 cases of *vivax* malaria treated with paludrine in doses of 20 to 1,500 mgm. per day for 14 to 28 days, however, the effect on the relapse rate was no greater than that of quinacrine.

We have studied the effect of paludrine against a standardized infection of a Southwest Pacific strain of *vivax* malaria in order to compare it with suppressive agents, and have attempted to determine whether or not it exhibits synergistic action with quinine or pentaquine.

PROCEDURE AND METHODS

General

¹ This investigation was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The studies were planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for the Coordination of Malarial Studies. This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General, U. S. Army.

Through a cooperative arrangement between Professor Clay G. Huff and Dr. Frederick Coulston, Department of Bacteriology and Parasitology, and the Malarial Research Unit, Department of Medicine, the former group bred *Anopheles quadrimaculatus* mosquitoes, supervised their infection and the inoculation of volunteers, and determined the intensity of infection in the salivary glands of the mosquitoes. The latter group assumed the responsibility for clinical care of patients studied by both groups.

The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, Inc., and Wyeth, Inc., for contributing toward the publication costs.

² Captain, M.C., A.U.S.

The subjects for the tests were presumably susceptible healthy, white, inmate volunteers in the Stateville Prison, which is located in a non-endemic area.³ All subjects were infected with Southwest Pacific *vivax* malaria (Chesson strain) (8) by the bites of ten infected mosquitoes, or by injection of their infected salivary glands.¹ To minimize the factor of acquired immunity, only primary attacks and early relapses were treated. Treatment with paludrine was started promptly after the demonstration of fever and parasitemia. A detailed report of the procedures used has been published (7).

The Chesson strain was chosen because it is characterized by a high relapse rate, a short latent interval between relapses, and a low incidence of delayed primary attacks (9, 10, 11). This strain has been used extensively in testing new antimalarial agents. It is possible under the conditions of these investigations to differentiate between subjects with severe infections and those with moderate infections, on the basis of the length of the prepatent period or the preceding parasitic latent period. In subjects with short preceding prepatent or latent periods the relapse rate of Chesson infections was 98 per cent after treatment with suppressive drugs,

³ The investigations reported in this paper would not have been possible except for the enthusiastic cooperation of the inmates and the administrative staff of Stateville Penitentiary.

whereas when these periods were long, the relapse rate was only 67 per cent (11).

Drug dosage and administration

In this report, the dosage of all drugs is given in terms of the weight of free base. All drugs were administered orally.

Paludrine. The daily dose of paludrine was 0.97 gram of base (equivalent to 1.11 grams of the monohydrochloride). The drug was administered to ten subjects in equally divided doses given at four-hour intervals throughout the 14 days of treatment. This dosage was chosen because earlier work of British investigators had demonstrated that 0.97 gram of the base per day produced minimal symptoms of toxicity. This dosage was the largest that had been used in man at the time our studies were begun, and was many times greater than the dosage which has been found effective in other strains of malaria (5, 6).

Throughout these studies the dosage and schedule of administration of paludrine have been the same whether it was administered alone or concurrently with other drugs.

Quinacrine (atabrine). The drug was administered in the form of the dihydrochloride. After an initial dose of 0.87 gram of the base (1 gram of the salt) given in four divided doses in the first 24 hours, the daily maintenance dose was 0.35 gram (0.4 gram of salt) given in four divided doses. Treatment was continued for seven days.

Pentaquine. Pentaquine was administered in the form of the monophosphate salt. The daily dose was 60 mgm. of the base given in single doses of 10 mgm. every four hours throughout the 14 days of therapy.

CHEMICAL METHODS

The concentration of paludrine in plasma was determined by the method of Spinks and Tottey (12); a modification (13) of the method of Brodie, Udenfriend, and Taggart (14) was used for pentaquine.

RESULTS

Comparison of paludrine with other suppressive agents. The efficacy of paludrine in terminating the acute attack of malaria was less than that of quinacrine, as evidenced by the number of days of parasitemia and the number of paroxysms with fever over 101° F. (rectal) after the start of treatment. Chloroquine (15) cleared the blood of parasites more rapidly than paludrine. Paludrine did not clear the peripheral blood of parasites in all patients until the seventh day of therapy whereas, in all patients treated with quinacrine, the thick film was negative in four days (Figure

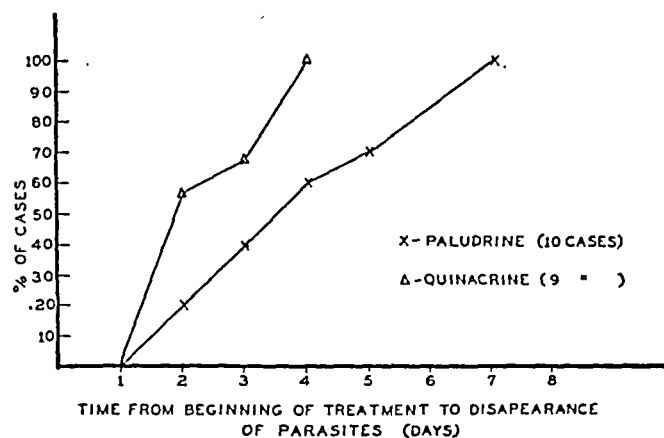


FIG. 1

1). All ten subjects treated with paludrine experienced one malarial paroxysm after treatment was started, and one subject had two paroxysms after the first day of treatment. No subjects treated with quinacrine had more than one paroxysm.

Seven of the ten subjects treated with paludrine relapsed after treatment; three subjects have shown no evidence of malaria during the 11 months which have elapsed since the end of therapy (Table I). Two of 22 subjects treated with quinine (15) failed to relapse, but all of the subjects treated with quinacrine or chloroquine (15) relapsed.

In no patients with severe infections was radical cure obtained when any one of these drugs was administered alone. The five subjects who failed to relapse after treatment with paludrine or quinine had infections of only moderate intensity (11).

The median period of parasitic latency following treatment with paludrine was slightly less than after quinacrine (Figure 2 and Table I). In four of the seven patients who relapsed after treatment with paludrine, parasitemia reappeared in 29 days or less. Parasitic relapse occurred within 34 days in half of the subjects who relapsed after treatment with quinacrine. After treatment with chloroquine (15) and after treatment with quinine (15), 60 days and 15 days, respectively, elapsed before parasitemia reappeared in half of the subjects.

The mean concentration of paludrine in the plasma during the period of therapy varied from 630 to 1,300 gamma per liter in individual subjects (Table II).

TABLE I

Therapeutic effect of paludrine administered alone and concurrently with other drugs

Drug	Dosage regime		Number of cases	Relapse ratio: Individuals relapsed Individuals treated		Duration of parasitic latent period in subjects who relapsed	Duration of follow-up in subjects who failed to relapse
	Total dose	Duration of therapy		Moderate infection	Severe infection		
	grams	days				days	months
Comparison of paludrine with quinacrine							
Paludrine	13.6	14	10	0/3	7/7	25, 28, 28, 29, 30, 34, 85	11, 11, 11
Quinacrine	3.0	7	9	2/2	7/7	29, 29, 31, 33, 35, 37, 37, 39, 43	
Effect of paludrine and quinine administered concurrently							
Paludrine and quinine	13.6 23	14	10	1/1	8/9	22, 25, 26, 34, 35, 36, 42, 62, 71	6
Effect of paludrine and pentaquine administered concurrently							
Paludrine and pentaquine	13.6 0.84	14	5		4/5	24, 29, 36, 120	6
Pentaquine	0.84	14	4		2/4	12, 128	11, 11

Paludrine was well tolerated at this dosage. Three subjects complained of mild transient abdominal discomfort during the first few days of therapy, and one subject complained of slight blurring of vision on occasions. No other subjective or objective evidence of toxicity was demonstrated by routine studies for drug toxicity (7).

Concurrent administration of paludrine and quinine. In order to determine whether or not there is a synergistic effect when paludrine and quinine are administered concurrently, ten subjects were treated during their primary attacks of *Chesson vivax* malaria with 0.97 gram of paludrine base and 1.6 grams of quinine base (2 grams of the

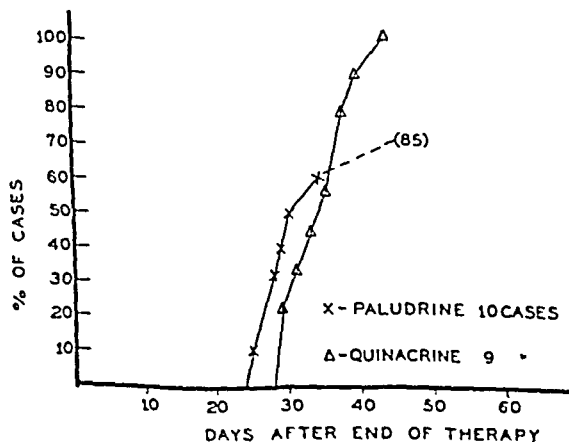


FIG. 2

TABLE II

Plasma concentrations of paludrine and concurrently administered drugs

Drug regime*	Range of mean concentration of paludrine in plasma	Range of mean concentration of concurrent drug in plasma
Paludrine alone	gamma/L. 630-1300	mgm./L.
Paludrine and quinine	770-1200	6-13
Quinine alone		3-10
Paludrine and pentaquine	650-1400	gamma/L. 140-450
Pentaquine alone		29-49

* All drugs were administered for 14 days in the following total dosage of base: paludrine 13.6 grams, quinine 23 grams, and pentaquine 0.84 gram.

sulfate) daily for 14 days (Table I). All patients experienced one paroxysm after therapy was instituted, and five to six days were required to clear the peripheral blood of parasites. The efficacy of this regime in terminating the acute attack of malaria was no greater than that of the same dose of quinine given alone (Table I). Nine of the ten subjects relapsed after therapy and one subject has shown no evidence of malaria for six months. In the nine subjects who relapsed, the parasitic latent period was no greater than that which followed treatment with the same dose of paludrine alone. The toxicity occasioned by this regime was mild, consisting of moderate abdominal pain, occasional vomiting, and diarrhea, plus mild cinchonism. The toxicity of the combined regime was therefore slightly greater than that produced by either drug when administered alone. There was no evidence of enhancement of the antimalarial effect of either drug by the concurrent administration of paludrine and quinine.

Concurrent administration of paludrine and pentaquine. Pentaquine (SN-13,276), a chemical analogue of pamaquin (plasmochin), has been shown to be superior to the latter as a curative agent in *vivax* malaria. The curative effect of pentaquine is enhanced by the concurrent administration of quinine (16). To determine whether concurrent administration of paludrine would also enhance the curative activity of pentaquine, a group of five subjects, whose previous history indicated that their infection presented a severe therapeutic challenge in tests for curative effect, were treated with paludrine and pentaquine concurrently.

The 14-day mean concentration of paludrine in the plasma, ranged from 650 to 1,400 gamma per liter. This was not different from the plasma concentration produced by the same dose of paludrine alone (Table II), or in combination with quinine. The 14-day mean concentration of pentaquine ranged from 140 to 450 gamma per liter in the subjects treated with pentaquine and paludrine, while in the control subjects treated with pentaquine alone, the plasma concentration varied from 29 to 49 gamma per liter. It is apparent that concurrent administration of paludrine increased the plasma concentration of pentaquine to a very marked degree. The mechanism responsible for this effect is unknown. It is of interest to note

that quinacrine has a similar effect on the concentration of pamaquin in plasma (17).

The effectiveness of the combined regime of paludrine and pentaquine in terminating the acute attack of malaria was not greater than that of pentaquine alone. Four of the five subjects treated concurrently with pentaquine and paludrine relapsed after treatment as did also two of four subjects treated with pentaquine (Table I). Only three of 17 patients presenting a severe challenge to the drug have relapsed following concurrent administration of pentaquine and quinine (16).

Despite the fact that concurrent administration of paludrine greatly increased the concentration of pentaquine in the plasma, it did not enhance the curative effect of pentaquine. Paludrine apparently does not manifest the synergistic effect on pentaquine which is exhibited by quinine.

CONCLUSIONS

Although paludrine fails to prevent relapse in the severely infected individual, it is a valuable drug for the treatment of the acute attack of *vivax* malaria (Chesson). It has little or no toxicity at doses much higher than the minimum necessary to suppress an attack; it does not stain the skin; and it is followed by a latent period longer than that after quinine and only slightly shorter than that after quinacrine (atabrine). Its prolongation of the subsequent latent period, however, is considerably less than that of chloroquine.

Concurrent administration of paludrine and quinine does not enhance the value of either in the treatment of malaria.

There is a marked increase in the plasma concentration of pentaquine when it is administered concurrently with paludrine, but there is no evidence of synergistic activity.

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A LICHEN-PLANUS-LIKE ERUPTION OCCURRING DURING THE COURSE OF CHLOROQUINE ADMINISTRATION¹

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During the course of studies of the chronic toxicity of chloroquine (SN-7618) in a group of 30 individuals, two exhibited lichen-planus-like eruptions. Since similar lesions have been described following quinacrine administration, detailed protocols of these two cases are herewith presented.

Chloroquine, [7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline], an antimalarial drug superior as a suppressive and therapeutic agent to both quinacrine (atabrine) and quinine (1, 2, 3), was administered to 30 inmate volunteers at the Illinois State Penitentiary at Stateville, Illinois, for one year in dosages in excess of that required for suppression (1) in order to test the chronic toxicity of the drug (4).³

Before medication was started, detailed histories and physical examinations were recorded; and clinical laboratory studies including tests of renal and hepatic function, blood counts, and electrocardiograms were done. During the observation period, the patients were periodically examined,

weighed, and interviewed and the laboratory tests were repeated.

Of 30 individuals, two developed cutaneous eruptions which resembled lichen planus. Drug administration in both individuals, as in the other members of the group, consisted of a full year of weekly oral doses of 0.5 gram of chloroquine.⁴ The concentration of chloroquine in the plasma reached a weekly peak of between 150 and 250 gamma per liter; and the weekly low level, taken just prior to the succeeding dose, ranged between 20 and 40 gamma per liter.

CASE REPORTS

Case 1: A 21-year-old otherwise healthy Negro male had had intermittently for several years dry circumscribed neurodermatitis on the nape of the neck. During the first eight months of the study, his only noteworthy symptoms were occasional headaches after taking chloroquine and occasional difficulty in rapid visual accommodation.

In the eighth month of treatment there appeared on the trunk, a faint macular rash which suggested tinea versicolor, but no fungi were found in scrapings on direct microscopic examination. In the eleventh month there was more pronounced scaling of the lesions. At that time the eruption consisted of brown, sharply demarcated macules with branny scales, over the shoulders and trunk, mainly on the back. Mild pruritus was present. The condition somewhat resembled both seborrheic dermatitis and pityriasis rosea. The following month (four months after its first appearance and in the twelfth month of chloroquine administration) the rash spread and became papular and infiltrated.

It then occupied the upper extremities, with a predilection for the flexor surfaces (Figure 1). There were scattered lesions on the medial aspects of the thighs and on the trunk, but none above the shoulders nor on the buccal mucous membranes or genitalia. The lesions varied in size from a few millimeters to several centimeters in diameter. The smaller lesions consisted of reddish violaceous papules with a smooth and slightly shiny surface. The larger lesions were annular and superficial, the center being depressed and paler than the periphery

⁴ Dosage is expressed in terms of base rather than salt.

¹ This investigation was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The work was planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for Coordination of Malarial Studies. This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General, U. S. Army.

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² Captain, M.C., A.U.S.

³ The investigations reported in this paper would not have been possible except for the enthusiastic cooperation of the inmates and the administrative staff of Stateville Penitentiary.

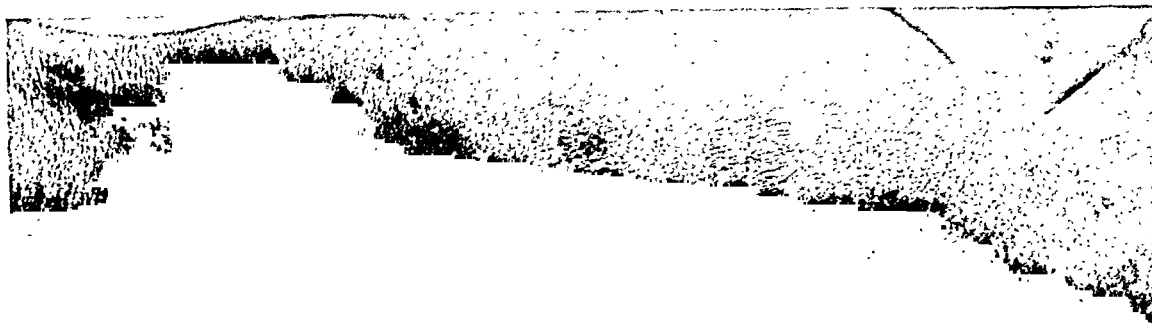


FIG. 1. CUTANEOUS ERUPTION DURING CHRONIC CHLOROQUINE ADMINISTRATION, CASE 1

The lesions scattered from shoulder to antecubital fossa vary from individual papules to large macules. The wrinkling of the central surface of the macules and the peripheral papular elevation are well shown.

which consisted of a ring of confluent, shiny, brownish-red or violaceous-red, infiltrated papules. Fine adherent scaling was present on the edges of some macules. None of the lesions was polygonal and no Wickham's striae were noted. The patient felt perfectly well during the period of the cutaneous eruption. There was no other evidence of systemic disease or drug intoxication.

The lesions began to subside within three weeks after

the last dose of chloroquine and continued to fade until, in five weeks, only macules of pigmentation with slight central depigmentation remained. Some of the macules showed slight wrinkling of the surface.

Biopsy of a typical lesion showed on microscopic examination (Figure 2) slight hyperkeratosis, but an otherwise normal pigmented epidermis. There was a dense perivascular lymphocytic infiltrate around the vessels of



FIG. 2. CUTANEOUS ERUPTION DURING CHRONIC CHLOROQUINE ADMINISTRATION

Photomicrograph of biopsy specimen in Case 1. Scattered lymphocytic infiltrates in the subpapillary layer and in the mid-corium.



FIG. 3. CUTANEOUS ERUPTION DURING CHRONIC CHLOROQUINE ADMINISTRATION

Photomicrograph of biopsy specimen in Case 2. Perivascular and periglandular round cell infiltration.

the subpapillary venous plexus. The microscopic appearance was that of mild chronic dermatitis.

Case 2: A 25-year-old healthy white male complained of no symptoms during the year of chloroquine administration except occasional headaches, often occurring six to 12 hours after the weekly dose of the drug and, on several occasions, of a transient urticaria.

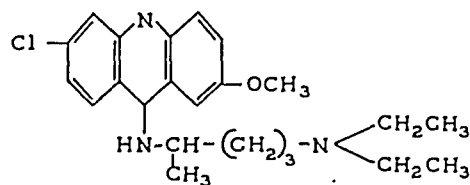
In the 12th month of chloroquine administration, a macular eruption appeared on the flexor surfaces of both arms. The macules were vivid red to faint yellowish red, the color completely fading on pressure under glass. No palpable lesions developed from the macules. They were not pathognomic of any skin disease and disappeared within six weeks after the drug was discontinued.

A biopsy of one of the lesions showed slight hyperkeratosis but an otherwise normal epidermis. There was a scattered lymphocytic infiltrate in the papillae, which were markedly edematous. In the subpapillary layer there was dense infiltration around the vessels of the subpapillary venous plexus. In one area in the lower corium there was a dense lymphocytic infiltrate around a sebaceous gland. The microscopic appearance (Figure 3) was reminiscent of that of lichen planus and similar to that of the eruption following quinacrine administration.

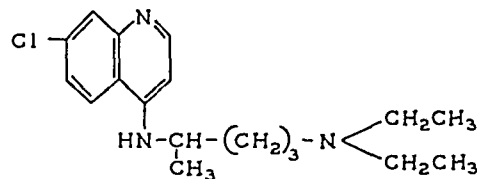
DISCUSSION

Two men who received 0.5 gram of chloroquine weekly for one year developed lichenoid skin eruptions. It may be only a coincidence that both of the individuals had evidence of other cutaneous disorders, namely, neurodermatitis in Case 1 and urticaria in Case 2. In Case 1, the eruption appeared in the eighth month as a macular rash and slowly developed into an infiltrated eruption; in Case 2, the macular eruption appeared only in the last month of chloroquine administration. Since in both instances the rash rapidly subsided after the drug was discontinued, it is possible that in Case 2 the eruption was aborted after the macular stage and might have progressed to palpable infiltration and desquamation like Case 1, had chloroquine been continued longer.

The eruption in Case 1 was definitely lichenoid, yet not a single lesion showed the characteristic polygonal shape or the Wickham striae seen in lichen planus papules. The microscopic appearance of the biopsy section in Case 1 was that of mild chronic inflammation. Yet in Case 2, although the clinical eruption did not resemble lichen planus, the microscopic appearance did. It should be emphasized that the clinical course in both cases was mild, subjective symptoms were minimal, constitutional symptoms absent, and the eruptions faded promptly when drug was discontinued.



QUINACRINE



CHLOROQUINE

FIG. 4. STRUCTURAL FORMULAE OF QUINACRINE (ATABRINE) AND CHLOROQUINE, SHOWING SIMILARITY OF CONFIGURATION

A lichen-planus-like eruption not infrequently complicated quinacrine (atabrine) administration to troops overseas (5, 6, 7, 8, 9). Individuals with lichen-planus-like eruptions due to quinacrine have been successfully treated for malaria with chloroquine without causing exacerbation of their skin lesions (3). However, one instance of exacerbation of a quinacrine-induced dermatitis upon the administration of chloroquine has been observed (10).

The development of a lichen-planus-like eruption as a toxic manifestation of chronic chloroquine administration has not been previously described though the drug has been administered to more than 5,000 individuals (1). It may be irrelevant that our patients had higher dosage than that required for suppression, since in 20 men in the toxicity study at Stateville who took 0.5 gram daily for 11 weeks, or seven times the suppressive dose, no cutaneous eruption was observed (4).

The occurrence with chloroquine of a lichen-planus-like eruption similar to that seen with quinacrine is of interest because of similarities in their chemical structures (Figure 4).

CONCLUSIONS

Of 30 healthy volunteers who were given chloroquine for one year in a dosage in excess of that required for antimalarial suppression, two developed cutaneous eruptions simulating the rash oc-

asionally caused by quinacrine. The patients had no serious systemic symptoms and the eruptions disappeared promptly when the drug was discontinued.

ADDENDUM

Three months after the end of chloroquine administration the drug was readministered to Case 1 at a dose of 0.3 gram a day for 17 days without reappearance of the eruption. Two months after the termination of the chronic toxicity study, Case 2 resumed taking chloroquine in a dose of 0.3 gram twice a week. No cutaneous eruption developed in six months' drug administration.

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STUDIES ON THE CHRONIC TOXICITY OF CHLOROQUINE (SN-7618)¹

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Chloroquine, 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline, is an antimalarial drug of the 4-amino quinoline series. It has been shown to have antimalarial activity similar to and superior to that of quinacrine in avian malaria, and in trophozoite- and sporozoite-induced *vivax* and *falciparum* malaria in man (1, 2, 3, 4).

The present study was undertaken for the purpose of establishing whether chloroquine can be administered for prolonged periods as a suppressive drug without causing serious toxicity. To accentuate toxic manifestations and establish the margin of safety, larger dosages than those necessary for suppressive treatment were administered. Normal inmate volunteers at the Illinois State Penitentiary at Stateville served as subjects for these investigations.

PROCEDURES AND METHODS

The subjects under study were selected on the basis of history and physical examination to exclude any volunteer with physical disability or neurosis. The following observations and tests were made prior to initiating treatment and were repeated at frequent intervals during the early

course of medication and later at gradually increasing intervals:

<i>General</i>	<i>Kidneys</i>
Subjective symptoms	Urinalysis
Oral temperature	Blood non-protein nitrogen
Weight	Phenolsulfonphthalein excretion
Physical appearance	<i>Nervous System</i>
<i>Cardiovascular System</i>	Visual acuity
Pulse rate	Diplopia
Blood pressure	Visual accommodation (monocular)
Electrocardiogram	Nystagmus
<i>Blood</i>	Extraocular movements
Erythrocyte count	Tremor
Leucocyte count, total and differential	Coordination and gait
Hemoglobin	Handwriting
<i>Liver</i>	Romberg test
Urine urobilinogen	Deep reflexes
Serum bilirubin	
Cephalin-cholesterol flocculation	

Toxicity studies were begun simultaneously on two groups of 20 subjects each. The first group was placed on a weekly-dosage schedule of 0.5 gram of chloroquine base, administered orally once a week. The second group was given a total daily dosage of 0.3 of the base in two doses (0.1 and 0.2 gram) for 77 days. At the end of this period the volunteers in the latter group joined those on the weekly-dosage regime at 0.5 gram (base) once a week, and both groups continued on this schedule for a total time of one year. Placebos were given for one week before and for two months after the year of chloroquine administration. During the periods of placebo administration no change was made in the routine of observation.

Because of parole, discharge, or for administrative reasons, 10 men failed to complete the full year.

Chemical method. The drug was determined in plasma by the method of Brodie *et al.* (5) with the following modifications: 30 ml. of heptane and 2 ml. of ethyl alcohol (charcoal treated and redistilled until non-fluorescent) were placed in a 60-ml. glass stoppered bottle. Ten ml.

¹ This investigation was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The work was planned in cooperation with the Panel on Clinical Testing of Antimalarials of the Board for Coordination of Malarial Studies. This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General, U. S. Army.

The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, Inc., and Wyeth, Inc., for contributing toward the publication costs.

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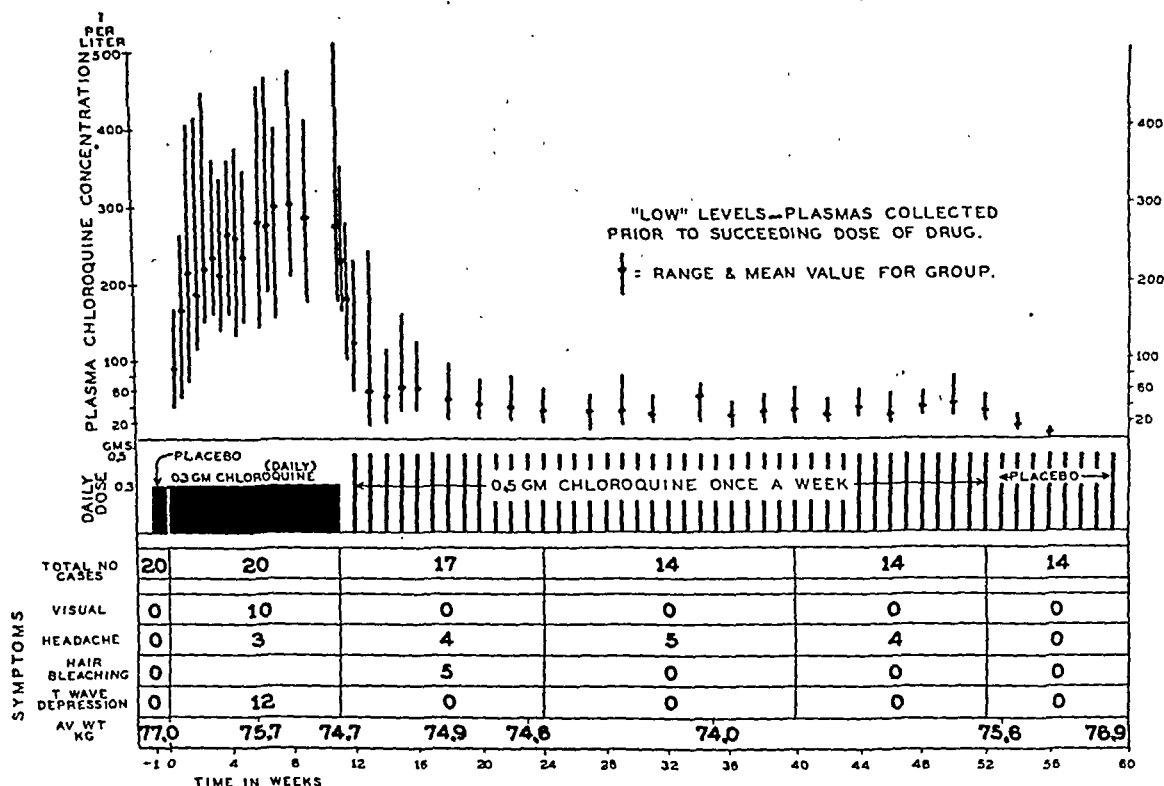


FIG. 1. TOXICITY OF CHLOROQUINE

Daily-dosage study. Plasma concentrations represent range and mean of the "low" levels; plasmas were drawn prior to the succeeding dose of the drug. The body weights are the average of only 12 cases, those lacking complete records being excluded. Drug dosages are expressed in terms of the base.

of plasma and 5 ml. of 0.2 N NaOH were added and the mixture shaken for 15 minutes. After the two phases separated, the water phase was aspirated and the heptane layer was poured into a 125-ml. glass-stoppered bottle containing 50 ml. of 0.1 N NaOH and shaken for 10 minutes. After the phases separated, the water phase was aspirated. Washing with 0.1 N NaOH was repeated three times. Prior to the third washing, 1 ml. of the non-fluorescent ethyl alcohol was added. After the separation of layers, and the aspiration of the water phase, 20 ml. of the heptane phase were transferred by means of a pipette, just previously rinsed with the non-fluorescent alcohol, to a 40-ml. glass-stoppered pointed centrifuge tube containing 6 ml. of 0.05 N HCl. The mixture was then shaken for 10 minutes. The heptane layer was removed by aspiration and 5 ml. of the acid phase were transferred to a tall fluorometer tube containing 0.25 ml. of a 5 per cent cysteine solution. Two and one-half ml. of buffer solution (buffer: 6 volumes of 0.6 N sodium hydroxide and 5 volumes of 0.6 M boric acid in 0.6 N potassium chloride) were then added.

All tubes (blanks, standards and unknowns) were placed in an irradiator for two hours' treatment with ultra-violet light emitted by an H-4 mercury arc lamp. Since the distance from the light to all tubes was not

uniform, the tubes, after one hour of irradiation, were moved to the opposite side of the irradiator for the second hour of treatment.

The intensity of fluorescence of the samples was determined in a Coleman Photofluorometer, Model 12 A, using B-1-S and PC-1 filters.

RESULTS

Concentration of drug in the plasma. In the group of volunteers receiving 0.3 gram of chloroquine daily the drug plasma concentrations, prior to the morning dose, increased for the first four weeks and remained on a plateau thereafter (Figure 1). There was a wide individual range. The level usually varied inversely with the body weight of the subject. When the dosage was reduced (after 77 days), 11 weeks elapsed before the plasma concentrations in this group fell to the levels of the group in which subjects had received 0.5 gram weekly from the start of the experiment.

In the weekly-dosage studies the individual "low" values, collected just prior to the next dose,

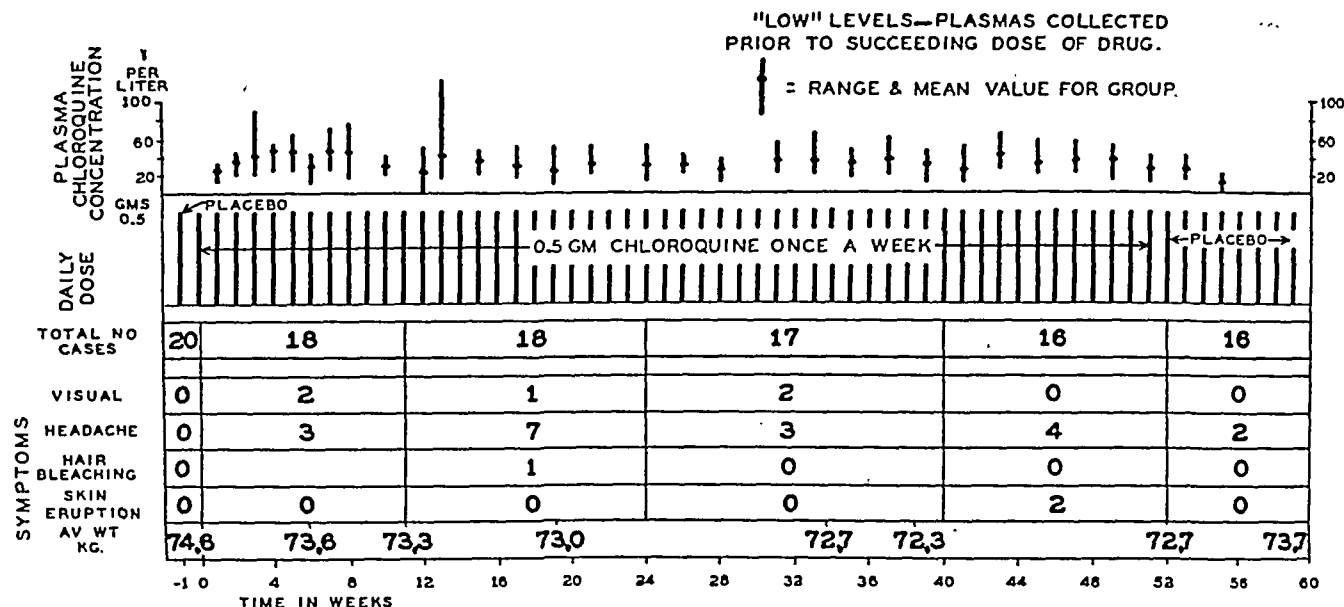


FIG. 2. TOXICITY OF CHLOROQUINE

Weekly-dosage study. Plasma concentrations represent range and mean of the "low" levels; plasmas were drawn prior to the succeeding dose of the drug. Body weights represent the average of those 14 individuals who completed the experiment. Drug dosages are expressed in terms of the base.

rarely fell below 20 gamma per liter and the means of these weekly values were usually between 25 and 40 gamma per liter (Figure 2).

During the first seven weeks of drug administration in the weekly-dosage group a record of the highest plasma concentrations was made by col-

lecting samples five hours after the weekly dose of 0.5 gram. The peak concentrations ranged from 44 to 346 gamma per liter. The mean peak concentrations for the group rose week by week, as follows: 89, 135, 176, 154, 197, 252, and 215 gamma per liter.

The rate of decline of the plasma concentration after one of the weekly doses is shown in Figure 3. In 48 hours the mean concentration (94 gamma per liter) was less than half of the six-hour level (215 gamma per liter). At seven days the mean had fallen to 29 gamma per liter.

Toxicity. The toxic phenomena encountered in the daily-dosage study were visual difficulties, bleaching of the hair, and electrocardiographic changes. Skin eruptions occurred in the weekly-dosage study. Some individuals in both groups lost weight and had headaches (Figures 1 and 2).

Visual symptoms were noted by half the subjects in the daily-dosage study but by only an occasional subject in the weekly-dosage group. These symptoms consisted of a difficulty in changing focus quickly from a near to a far object. The tests for visual acuity, power of monocular accommodation, and diplopia failed to demonstrate objective abnormality. However, no tests for speed of accommodation were performed. The visual symptoms in the daily-dosage group disap-

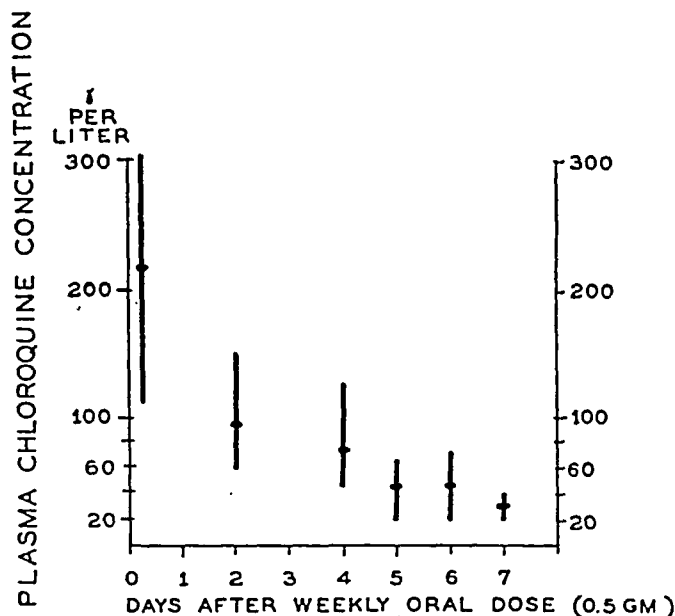


FIG. 3. PLASMA CHLOROQUINE CONCENTRATION

Means and ranges of plasma concentrations during seven days following termination of oral administration of 0.5 gram of chloroquine base to each of 18 individuals.

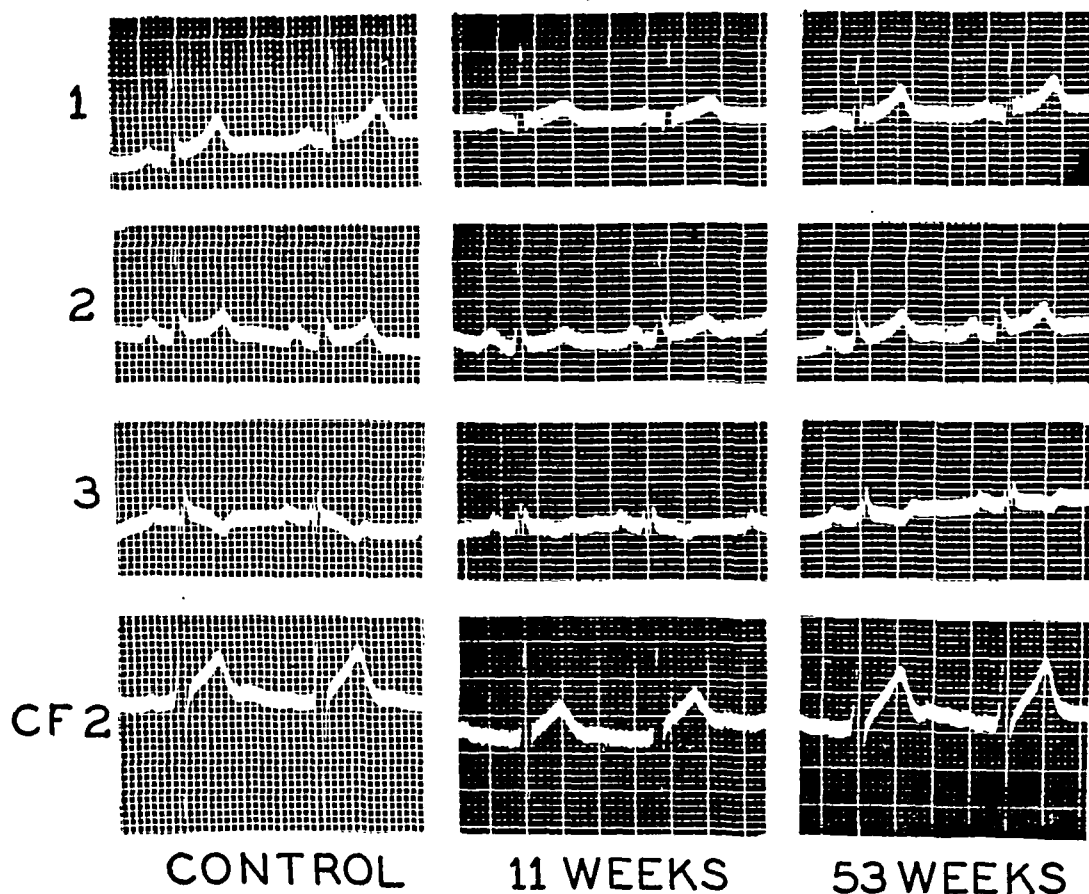


FIG. 4. TOXICITY OF CHLOROQUINE

Electrocardiograms taken after 11 weeks of chloroquine at 0.3 gram of base daily. Normal curves returned in spite of continued dosage at 0.5 gram of base weekly.

peared when the dosage was lowered to 0.5 gram weekly.

Shortly after the conclusion of the daily-dosage study, it was noticed that the hair of one of the blond subjects was lighter near the roots. Examination of others showed that bleaching had occurred in all blond subjects in the daily-dosage experiment and possibly in one of the two blond subjects in the weekly-dosage test. The hair gradually regained its normal color after reduction of the dosage in the former group.

Electrocardiographic changes were noted in 12 of the 20 men in the daily-dosage study. The changes consisted of a concordant diminution of the height of the T-waves in some or all of the leads. No other evidence of cardiovascular abnormality was observed, and the T-waves regained their original amplitudes after the dosage was reduced (Figure 4).

In two individuals in the weekly-dosage group, mild skin eruptions developed during the last few months of drug administration. The eruptions occurred over the flexor surfaces of the extremities and on the trunk. In one subject the lesions consisted of reddish violaceous papules and annular macules with a ring of papules about a paler center. The appearance resembled lichen planus, but no single lesion was typical of that disease. Histological studies showed only chronic inflammation. In the other subject there were reddish macules of similar distribution which on histological section were suggestive of lichen planus. The case histories of the two individuals with skin eruptions are presented elsewhere (6).

Both groups lost weight. The weight loss was slight but probably significant. At 38 weeks, the average weight had fallen 2.6 kg., most of which was regained in the placebo period. Of 26 indi-

viduals with complete weight records, 23 lost weight. Most of the weight loss in the daily-dosage group occurred during the 77 days when dosage was high and was not regained until the placebo period.

The appraisal of headaches was difficult. In the daily-dosage studies, headaches, different from those previously experienced, were noted by six of the 20 subjects. During the course of the weekly-dosage experiments, headaches were occasionally reported. Sometimes they recurred for several weeks in the same individual 6 to 12 hours after the drug was administered. Occurring in the occipital or frontal areas, or both, the headaches were usually mild, but lasted several hours and were occasionally still present the following morning. Only those headaches which seemed to be attributable to the drug by temporal relationships and history were included in Figures 1 and 2. Two subjects, however (Figure 2), continued to have headaches during the second placebo period.

The other tests and observations failed to reveal abnormalities.

DISCUSSION

The amount of chloroquine *base* recommended for the treatment of an attack of *vivax* or *falciparum* malaria is 1.5 grams in three days, and for suppression, 0.3 gram a week (1). The daily-dosage group received 23.1 grams in 77 days and the weekly-dosage schedule was 0.5 gram a week. In both studies, therefore, the dosage used was considerably in excess of that required for antimalarial therapy or suppression. This difference is further emphasized by a comparison of the plasma chloroquine concentrations attained in these studies with the concentrations required for antimalarial suppression. In the daily dosage studies mean "low" plasma concentrations in excess of 200 gamma per liter were maintained for 10 weeks while a plasma chloroquine concentration of 10 gamma per liter is adequate for antimalarial suppression (7). Individual "low" values in excess of 500 gamma per liter occurred without unusual symptoms.

The toxic manifestations in the group on the daily-dosage schedule were in fact reversible and caused no incapacity. There were unequivocal visual disturbances similar to those described by other observers (7, 8, 9, 10). The T-wave de-

pression in the electrocardiograms was similar to that reported as a result of drugs unrelated to chloroquine (11, 12, 13, 14, 15, 16). It was probably non-specific and without clinical significance. The bleaching of the hair gradually disappeared after the dosage was reduced, as it did in cases described by Butler (9).

The toxic symptoms which occurred in the subjects on the weekly-dosage regime were milder. It should be emphasized that the difficulty in accommodation was a common complaint in subjects who received 0.3 gram daily but it was very rare in those who received 0.5 gram once a week. The skin eruption which developed in two individuals was mild but similar to that reported during suppressive therapy with quinacrine. More extensive experience with chloroquine will be necessary to determine its incidence and seriousness. In both cases the rash faded rapidly after the year of treatment was completed.

The temporal relationship of headaches to medication suggested an etiological relationship, but their unpredictability in incidence, location, and severity made it impossible accurately to appraise this symptom even after a year of close observation. The loss of body weight was small and disappeared with discontinuance of medication.

The special tests performed gave no clue to the mechanism of the toxic effect of chloroquine.

SUMMARY AND CONCLUSION

1. Two groups of inmate volunteers of 20 each were given chloroquine (SN-7618) orally for one year in greater dosages than those required for antimalarial therapy or suppression, in order to detect and evaluate any toxic reactions.

2. The first group took 0.3 gram (base) daily for 77 days and 0.5 gram (base) once weekly thereafter. On the higher dosage, visual disturbances, headache, bleaching of the hair, electrocardiographic changes, and slight weight loss were observed. These changes caused no incapacity and diminished or disappeared when the dosage was decreased.

3. The second group, which received 0.5 gram (base) weekly from the beginning of investigations, had occasional headaches, slight weight loss, and, in two cases, a skin eruption resembling lichen planus.

4. Under the conditions of this investigation, it can be concluded that chloroquine is a safe antimalarial compound when given in the recommended dosage.³

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

I. METHOD FOR THE QUANTITATIVE ASSAY OF SUPPRESSIVE ANTIMALARIAL ACTION IN VIVAX MALARIA^{1, 2, 3}

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INTRODUCTION

The wartime program for the development of new antimalarial drugs made apparent the need for satisfactory techniques for the assay of their activities in the human malarias. It seemed likely that the use of blood-induced malaria would partly satisfy this need. Information which has accrued since the beginning of these studies now permits the interpretation of results obtained with this type of infection in terms of a reasonable hypothesis concerning the biology of the malarias.

It is now believed that in mosquito-transmitted vivax malaria the sporozoites are localized initially in macrophage cells where they undergo growth, segmentation, and sporulation. This initial tissue phase yields forms of the plasmodia capable of invading and multiplying in the erythrocytes, thereby initiating a clinical attack of malaria. A portion of the tissue phase is believed to persist and to release periodically forms capable of erythro-

cytic invasion, thereby being responsible for the repeated, true relapses of vivax malaria. On the other hand, it is now quite clear that vivax malaria induced by the simple transfer of parasitized blood is devoid of a tissue phase and, therefore, does not relapse.

In accordance with this general view of the biology of vivax malaria, there are at least three different types of antimalarial activity (1). Activity is considered to be *prophylactic* if the action is exerted against the sporozoites or the parasites of the initial tissue phase of the disease; *suppressive*, if against the parasites of the asexual erythrocytic phase of the disease; and *curative*, if against the parasites of the persisting tissue phase. Since the primary need in wartime was for agents with suppressive activity, i.e., with a quinine- or quinacrine-like action, it was believed that the blood-induced infection, consisting entirely of the erythrocytic phase of the plasmodium, afforded the best test object with which to evaluate this type of antimalarial activity.

Therefore, studies were undertaken to determine whether blood-induced malaria is suitable for the quantitative appraisal of drug activity, to compare the susceptibility of erythrocytic parasites derived from simple blood transfer and from sporozoite inoculation, and to determine whether information obtained with a single strain of plasmodium is applicable to problems involving the treatment of malaria due to other strains.

BLOOD-INDUCED MCCOY STRAIN VIVAX MALARIA

Material and methods

The comparative assay of potential antimalarial drugs requires a highly standardized infection and testing procedure. The reliability of the therapeutic test depends largely upon a uniform host-susceptibility and the use of

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A part of the material in this paper has been presented in a Harvey Lecture by Dr. James A. Shannon (1) and also appeared in "Survey of Antimalarial Drugs, 1941-1945," p. 177, J. W. Edwards, Ann Arbor, Mich., 1946. Permission to use Tables I, II, IV and VI and Figures 1, 2, 3 and 4, has been obtained from the Harvey Society and from the editors of the Survey.

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an infective organism with stable characteristics. These considerations are important in the selection of experimental subjects and in the design of the therapeutic test.

The subjects used were individuals with central nervous system syphilis who presented no medical contraindications to induced malaria. Special attention was directed, in obtaining the history, to the place of birth, racial extraction, residence in malarious areas, and previous malaria. Only those white patients who had never lived in a malarious area and who gave no history of previous malaria were considered suitable for vivax infections. These criteria specifically exclude all colored races and all persons born in Italy, the Eastern Mediterranean countries, Central and South America, and the West Indies, in addition to other recognized malarious areas.

These studies were performed with the McCoy strain of *P. vivax* originally isolated by Boyd in 1931 (2). The character of the infection which it produces has been the subject of extensive studies by Boyd and his associates (3). The off-shoot of the strain used in the present work has been in continuous human passage by blood transfer since 1936.

To insure maximal stability of the strain in its virulence and response to chemotherapeutic agents, its passage was restricted to individuals presumed to be completely susceptible and was performed before the beginning of therapy, *i.e.*, before the fourth or fifth day after the onset of fever. Exposure of the strain to acquired immune bodies in the host, which might in time modify the characteristics of the parasite, was thereby minimized. Transfer prior to therapy removed the theoretical hazard of developing drug resistance.

Biological characteristics of blood-induced McCoy vivax malaria

The biological characteristics of blood-induced McCoy vivax malaria will be described briefly in terms of parasitemia and fever. The number of parasites in the circulating blood is generally too low, during the first few days after inoculation with 500,000 parasites, to permit their ready demonstration by the usual thick blood smear technique. This *prepatent* period varies from two to 12 days, with an average of about six days. The onset of fever usually bears a close relationship with the appearance of a demonstrable number of parasites in the blood. Thus, on the first day of fever above 100.6°, a negative thick smear is found in about one-quarter of non-immune individuals and the parasite count is only occasionally above 1000 per cu. mm.

The first week of a primary attack is characterized by an irregular, sustained fever of moderate degree and a rapidly increasing density of parasites in the blood. By the fourth day of fever, a

negative thick smear is a rare finding and approximately one-half of the individuals show parasite counts in excess of 1000 per cu. mm.

During the second week of an uninterrupted infection, the fever assumes the usual intermittent pattern of malaria with regular tertian or quotidian paroxysms. The schizogonous cycle covers a period of 40 hours. The parasite count tends to become relatively stable between 2000 to 10,000 per cu. mm., although it has been observed to rise to as high as 75,000.

The spontaneous termination of fever in the uninterrupted infection usually occurs between the 12th and 25th days after the onset, the average being 17 days. Although the parasite count may fall rather rapidly at the time of spontaneous termination, positive thick blood smears persist for a period of days or weeks thereafter. Spontaneous termination has not been observed earlier than the 12th day in any white patient with a completely negative history of previous contact with the disease.

Therapeutic interruption of the infection early in its course may modify, in certain respects, a second experience with the disease. The onset of fever during a recrudescence, or following reinoculation, usually coincides with a higher density of parasites than was the case during the primary attack. This increased tolerance to the parasite may be interpreted as a manifestation of immunity acquired during the initial bout of fever. It would appear that the development of immunity during a period of clinical latency following therapy is not sufficient to render the patient resistant to a second infection, since interruptions up to 30 days in length do not diminish the average total duration of fever by more than two or three days.

Routine of the therapeutic test

Each subject was inoculated with 500,000 parasites from a patient in the fourth or fifth day of fever. Blood smears were obtained daily throughout the period of observation. The usual thick blood smears were used until the parasite count reached approximately 50 per cu. mm. Thereafter, a technique (4) involving the delivery of a definite volume of blood onto a measured area of the slide was utilized.

Therapy was started on the fourth or fifth day after the onset of fever above 100.6° (rectal). The test drug was administered by a regimen designed to achieve a fairly stable plasma drug concentration of the desired level for four days. Each course of therapy was in-

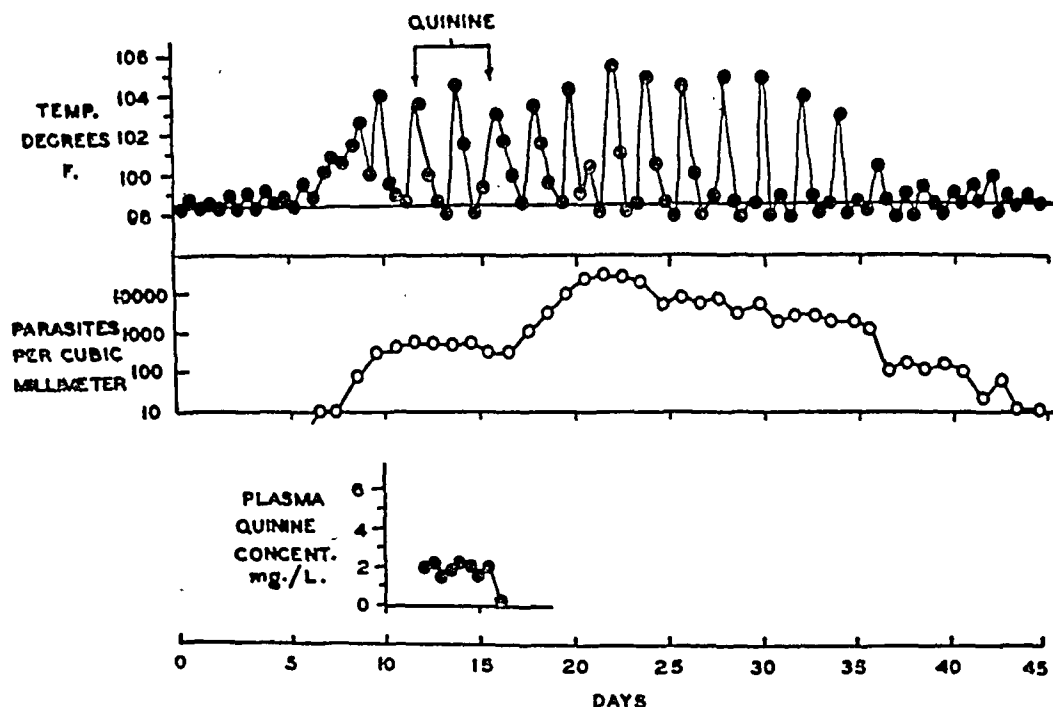


FIG. 1. CLASS I RESULT—NO EFFECT

initiated by a priming dose and continued with smaller doses at four- to six-hour intervals; all doses are recorded in terms of quinine base. Blood samples for the estimation of the plasma quinine concentration (5) were obtained at sufficiently short intervals to permit an appraisal of the mean concentration each day. The mean plasma drug concentrations referred to throughout this paper are the averages of the individual daily mean concentrations.

The observation period subsequent to therapy began on the day following the last effective plasma drug level. In the event of a complete disappearance of parasites and fever, the patient was observed for at least 14 days. If, at the end of this period, there had been no evidence of renewed activity of the infection, reinoculation with one million parasites was performed and the patient followed for an additional 14 days.

Classification of therapeutic results

The therapeutic results have been classified in three groups on the basis of the course of the parasitemia and fever following therapy.

Class I (No effect). The results falling into this group are those in which the administration of drug had no certain effect upon either the parasite density in the blood or the course of the febrile paroxysms (Figure 1).

Class II (Temporary effect). This group includes those results in which there was at least a temporary, partial, or complete, suppression of parasitemia or fever. Disappearance of fever and parasites, followed by spontaneous recurrence

within 14 days after the last effective plasma drug level, was considered to be a complete, temporary effect (Figure 2). If the parasite count on the fifth day after starting therapy was less than half the maximum count during drug administration, and subsequently increased, the result was classified as a partial effect. A reduction of the febrile elevation to less than half of that of the preceding and subsequent paroxysms was also considered to be a partial effect.

Class III (Permanent effect). The therapeutic results fall into this group when parasites were absent from thick blood smears for at least 14 days after the last effective plasma drug level and when continued susceptibility of the host to the infection was established by the appearance of parasitemia and fever following reinoculation (Figure 3).⁴

⁴ A review of the data on patients in whom reinoculation was delayed beyond the usual observation period indicates that an occasional individual may manifest a spontaneous recurrence after the 14th day. Since prepatent periods of less than two days have not been observed in patients receiving inocula of the size used in these studies, it has been assumed that parasitemia observed within 48 hours after reinoculation can be attributed to parasites persisting from the primary attack. These cases have therefore been placed in the Class II category of therapeutic effects. Such an occurrence has not been sufficiently frequent to prejudice the data on any given drug.

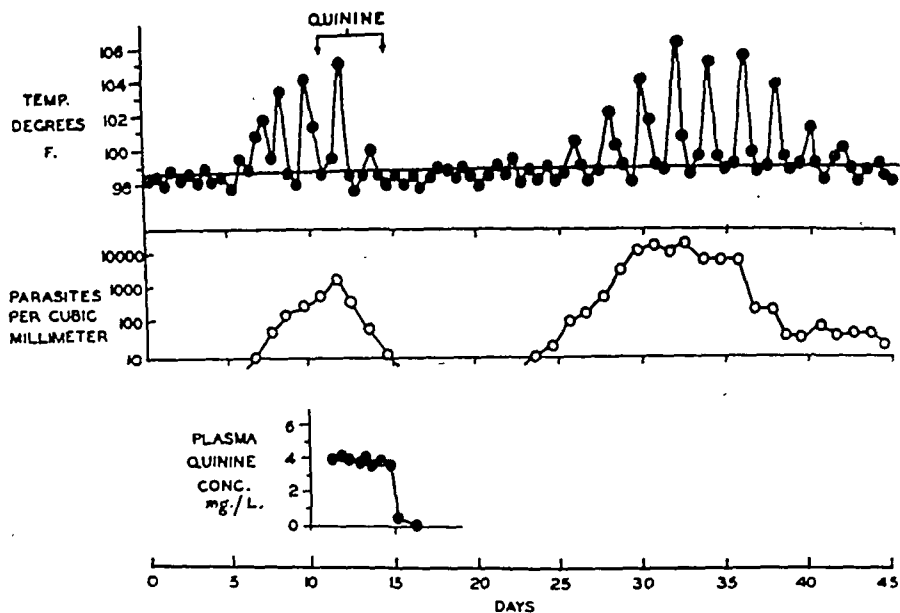


FIG. 2. CLASS II RESULT—COMPLETE TEMPORARY EFFECT

The susceptibility of blood-induced McCoy vivax malaria to quinine

The first series of observations (1942) involved the administration of quinine to 12 patients with blood-induced McCoy strain vivax malaria. The experimental results are summarized in Table I. The mean plasma quinine concentrations ranged from 1.4 to 6.2 mg. per liter. On the basis of the

criteria established for this test, it was found that plasma concentrations of 2.0 mg. per liter or less exerted no certain effect upon the course of parasitemia or fever (Class I). On the other hand, mean concentrations of 5.0 mg. per liter or higher consistently produced a permanent interruption of the infection (Class III). Plasma concentrations between 2.0 and 5.0 mg. per liter resulted in

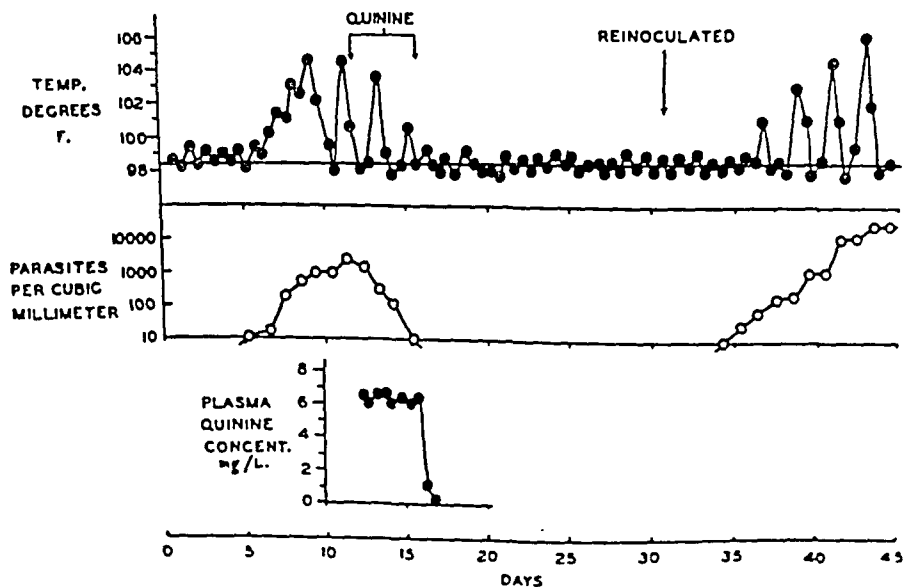


FIG. 3. CLASS III RESULT—COMPLETE PERMANENT EFFECT, POSITIVE REINOCULATION

TABLE I

The relationship between dosage and plasma concentration of quinine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

1942 series

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Dia	0.30	6.2			x
Pol	0.30	5.8			x
Mes	0.30	5.1			x
Fat	0.20	4.3			x
Bai	0.30	3.9		x	
Lyn	0.17	3.7		x	
Def	0.24	3.0		x	
Gol	0.10	2.5		x	
Mac	0.10	2.3		x	
Sic	0.10	2.2	x		
Sha	0.10	1.8	x		
Car	0.10	1.4	x		

Class II effects, temporary suppression or interruption of parasitemia and fever.

A series of similar observations was obtained in 18 subjects one and a half years later (Table II). The same critical plasma quinine concentrations were found to divide the three classes of therapeutic effect. These data indicate that the

TABLE II

The relationship between dosage and plasma concentration of quinine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

1943 series

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Irr	0.30	8.9			x
Gue	0.30	8.5			x
Klo	0.47	6.5			x
Gre	0.47	6.1			x
Set	0.30	6.1			x
Bal	0.38	5.0			x
Pin	0.30	5.0		x	
Ker	0.39	4.1			x
Mau	0.30	3.5		x	
Tar	0.30	3.4		x	
Hop	0.36	3.0		x	
Tsu	0.10	2.9		x	
Mor	0.10	2.9		x	
Don	0.30	2.9		x	
And	0.43	2.9		x	
Cic	0.30	2.8		x	
Hun	0.10	1.9	x		
Jam	0.10	1.2	x		

susceptibility to quinine is a stable characteristic of the infective organism.

The relationship between mean plasma quinine concentration, maintained for four days, and the therapeutic result is sufficiently consistent to permit the definition of a critical plasma drug concentration above which permanent interruption of the erythrocytic phase may be expected. The minimal quinine concentration capable of producing a detectable suppressive action may also be defined from data obtained with this testing procedure.

The correlation between the daily oral dose of quinine and the therapeutic effect is not as close

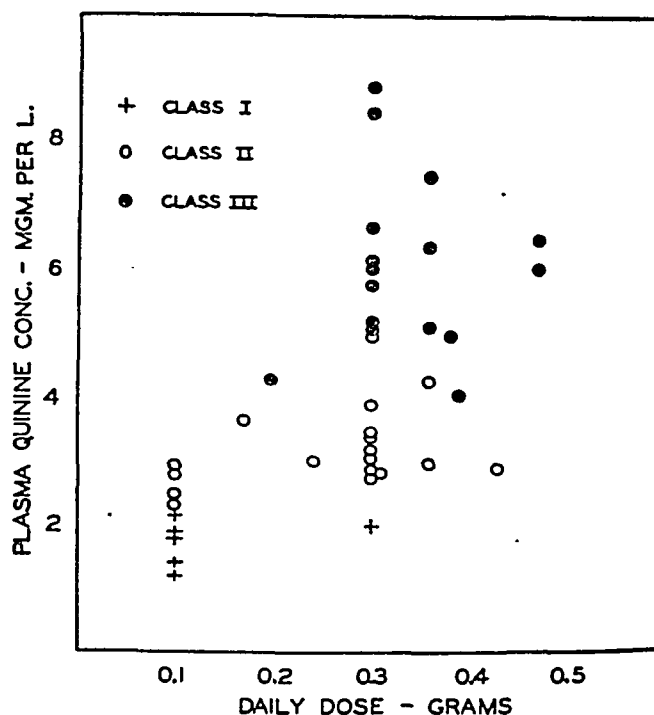


FIG. 4. RELATION BETWEEN DAILY ORAL DOSE AND MEAN PLASMA QUININE LEVELS AND EFFECT IN FOUR-DAY TESTS AGAINST BLOOD-INDUCED VIVAX (McCOY STRAIN) MALARIA

as that between mean plasma quinine level and effect (Figure 4). This is presumably the result of the considerable variation in plasma quinine concentrations achieved by different individuals receiving the same dosage regimen. It may be noted, in Figure 4, that all three classes of therapeutic effect were obtained with total daily doses of 0.3 gram, the response being dependent upon the plasma quinine concentration achieved. It is apparent that a quantitative appraisal of the anti-malarial activity of quinine is more readily obtained on the basis of the mean plasma quinine

concentration than on the basis of oral dosage.

The relationship between the class of therapeutic effect and the mean plasma drug concentration is independent of the parasite density at the beginning of therapy (Table III). Thus, a given drug concentration produces the same therapeutic effect whether the initial parasite count is low or high in otherwise comparable individuals.

MOSQUITO-INDUCED MCCOY STRAIN VIVAX MALARIA

Several investigators have expressed the belief that blood-induced malaria is more susceptible to therapy than is naturally-acquired or sporozoite-induced malaria (6). Consequently, it was important to determine whether the erythrocytic phase of a given strain of *P. vivax* has the same susceptibility to chemotherapeutic agents when established by the injection of infected blood as when derived from the underlying tissue phase of the mosquito-induced infection. Were this true, the suppressive action of an antimalarial agent, as tested against blood-induced vivax malaria, would be a correct measure of its suppressive activity in the mosquito-induced disease.

TABLE III

Distribution of 109 consecutive subjects by the parasite count at the start of therapy and by the therapeutic effect obtained

Parasite count at start of therapy	Number of subjects with therapeutic effect			
	Class I	Class II	Class III	Total
<i>per cu. mm.</i>				
1-100	8	11	8	27
101-1000	10	15	10	35
1001-10,000	11	18	8	37
> 10,000	1	6	3	10
Total	30	50	29	109

The selection of patients, the technique for testing for antimalarial activity, and the criteria for interpreting results were the same for sporozoite-induced malaria as was described for the blood-induced infection, except for the mode of infection and the time of observation before reinoculation. Malaria was induced by the bites of *A. quadrimaculatus* mosquitoes infected with the McCoy strain of *P. vivax*.⁵ This strain had been transmitted by mosquitoes for several years prior to this study. Mosquitoes were applied between four and 14 days after be-

⁵ The infections were originally established on this Service by infected mosquitoes kindly furnished by Doctor Robert B. Watson.

TABLE IV

The relationship between dosage and plasma concentration of quinine and therapeutic effect in four-day tests against mosquito-induced McCoy vivax malaria

Patient	Inoculum*	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect			Interval between inoculation and first true relapse
				I	II	III	
		grams (base)	mg./L				days
Fad	5+	1.44	13.1			x	
Bla	2+	1.44	10.7			x	
Dav	10+	1.44	9.8			x	247
Kas	4+	1.44	9.3			x	244
O'B	3+	1.44	8.6			x	276
Sch	11+	0.36	7.5			x	
Lon	15+	0.30	6.7			x	260
Dot	7+	0.36	6.4			x	
Koz	9+	0.30	5.2			x	261
Sim	7+	0.36	5.1			x	
Sma	8+	0.36	4.3		x		234
Hol	15+	0.30	3.2		x		264
Pol	15+	0.30	3.1		x		
Kin	14+	0.30	2.9		x		
Bue	11+	0.30	2.0	x			167

* Summation of individual mosquito infection densities.

coming gland positive. Subsequent to biting, the salivary glands of the mosquitoes were removed by dissection and examined for the presence of sporozoites. The density of gland infections was recorded on the basis of 1 to 4 plus. The inoculum indicated in Table IV represents the summation of the infection-densities of the mosquitoes biting each patient. Reinoculation was performed after an observation period of 21 days rather than the 14-day period utilized in the blood-induced infections. Infected blood from patients with mosquito-induced McCoy vivax malaria was used for the reinoculations.

The relationship between plasma quinine concentration and therapeutic effect in sporozoite-induced McCoy vivax malaria was examined in 15 susceptible individuals (Table IV). The plasma quinine level which, when maintained for four days, results in Class III effects, was 5.0 mg. per liter. Quinine concentrations between 3.0 and 5.0 mg. per liter produced Class II effects, while there was no effect in one patient who had a mean plasma quinine concentration of 2.0 mg. per liter. Reinoculation after a parasite-free period of 21 days was invariably successful.⁶ The short time (two to nine days) between reinoculation and parasitemia and clinical malaria supports the

⁶ Contrary to experience with avian malarias, it is possible to produce an acute attack by the inoculation of erythrocytic parasites in patients who have persisting infections unaccompanied by demonstrable parasitemia.

belief that these recurrences were the result of the reinoculation and not true relapses of the original mosquito-induced malaria. The time of occurrence of subsequent true relapse is indicated in the last column of Table IV.

It was shown in the preceding section that the critical plasma quinine concentration for Class III effects is 5 mg. per liter for blood-induced McCoy vivax malaria in the standard therapeutic test. The mosquito-induced infection, when tested in a similar fashion, was found to yield Class III effects at essentially the same plasma quinine level. This level, which permanently interrupts the blood-induced infection, does not prevent the later relapses which are characteristic of mosquito-induced infections. These results are in accord with the belief that, unlike the blood-induced infection, mosquito-induced malaria is characterized by a persisting tissue phase which is not materially affected by quinine and which is capable of producing relapses.

The suppressive antimalarial activity of quinine thus would appear to be identical in blood- and mosquito-induced infections. It is logical to assume that a similar situation obtains in the case of other suppressive drugs.

SOUTH PACIFIC (CHESSON STRAIN) VIVAX MALARIA

Blood-induced infections

It has been shown that the erythrocytic phases of both blood- and sporozoite-induced vivax malaria of the same strain (McCoy) have the same susceptibilities to quinine. However, there is reason to believe that the erythrocytic phases of malaria due to other strains of *P. vivax* have different susceptibilities to chemotherapeutic agents (7). Therefore, the suppressive antimalarial effect of various plasma quinine concentrations against another strain of *P. vivax* was studied. This strain (Chesson), obtained from a soldier who contracted malaria in New Guinea in 1944, produces an infection characterized by frequent, repeated relapses which occur as early as one week after the termination of a full course of quinine therapy (8, 9).

Except for the strain of parasite used and the time of reinoculation, the routine of the therapeutic test was the same as that described for the blood-induced McCoy strain vivax infections.

TABLE V

The relationship between dosage and plasma concentration of quinine and therapeutic effect in four-day tests against blood-induced Chesson vivax malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	<i>grams (base)</i>	<i>mg./L</i>			
Lyn	1.50	12.8		x	
Ruf	1.50	12.5		x	
War	1.50	11.9			x
Hop	1.50	11.2			x
Wil	1.50	10.4			x
McP	0.36	5.5		x	
Kup	0.36	5.1		x	

The effect of four days of quinine therapy against blood-induced Chesson vivax malaria was studied in seven patients (Table V). It is apparent that the highest plasma quinine concentrations which can be achieved without undue discomfort to the patient do not consistently result in Class III, or "permanent," effects. Furthermore, two patients with mean plasma quinine concentrations of 5.5 and 5.1 mg. per liter, respectively, had only the slightest detectable effects. Plasma quinine levels of this magnitude, when maintained for four days, invariably result in Class III effects with the McCoy strain of *P. vivax*. Therefore, the duration of therapy was extended to six days and the effect of quinine examined in 10 patients (Table VI). Under these conditions, it was found that the critical plasma

TABLE VI

The relationship between dosage and plasma concentration of quinine and therapeutic effect in six-day tests against blood-induced Chesson vivax malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	<i>grams (base)</i>	<i>mg./L</i>			
Des	1.50	14.6			x
Sei	1.50	12.8			x
Ger	1.90	12.6			x
McL	1.90	11.8			x
Wen	0.70	11.5			x
Hur	1.50	8.6			x
Meh	1.90	8.0			x
Anz	0.30	6.6		x	
Oak	0.70	6.0		x	
Per	0.70	3.6		x	

quinine concentration for Class III effects falls in the range of 7 to 8 mg. per liter.

The data presented demonstrate that the erythrocytic phase of Chesson strain vivax is considerably more resistant to the action of quinine than is that of McCoy strain vivax. The complete eradication of the erythrocytic phase requires, on the average, a daily dosage of 2 grams quinine sulfate (equals 1.6 grams quinine base) for a minimum period of six days.

Mosquito-induced infections

The suppressive action of quinine could not be quantified in sporozoite-induced Chesson vivax malaria, since relapses occur during the usual period of observation preceding reinoculation. That the early recurrences of clinical activity following the termination of suppressive therapy are true relapses, and not simple recrudescences due to inadequate therapy, is indicated by the data in Table VII. All of these patients received quinine therapy considerably in excess of that required to eradicate the erythrocytic forms in blood-induced Chesson strain infections. Thus, in dealing with strains which characteristically produce relapses soon after termination of therapy with suppressive drugs, the use of blood-induced malaria is of special value in determining the resistance of the erythrocytic phase to chemotherapy.

DISCUSSION

The data presented indicate that the susceptibility of the erythrocytic phase of the plasmodium to quinine can be quantitatively and reproducibly described. A wide gradation of clinical response

TABLE VII

Interval between treatment and relapse in mosquito-induced Chesson vivax malaria treated with quinine

Number of patients	12
Daily dose of quinine	1.5 grams (base)
Duration of therapy	16 days
Interval from end of therapy to appearance of parasitemia	days
Primary attack	
Range	5-9
Mean	8
First relapse	
Range	6-13
Mean	9
Second relapse	
Range	6-18
Mean	12

has been demonstrated to bear a striking relationship to the mean plasma quinine concentration maintained during the therapeutic period. Of greater importance is the fact that the use of plasma quinine concentrations permits a precise definition of antimalarial activity with relatively few experimental subjects. *A priori*, one may expect a similar situation to obtain in the examination of the suppressive activities of other agents.

It has been demonstrated that the erythrocytic forms derived from blood- and sporozoite-induced infections possess an equal susceptibility to quinine. This finding implies that the susceptibility of erythrocytic forms to quinine is a stable characteristic of the strain of the plasmodium. The long-accepted belief that blood-induced malaria is unusually susceptible to chemotherapeutic agents would appear to be the result of studies in which inadequate consideration was given to the degree of acquired immunity in experimental subjects, the differentiation of simple recrudescence from true relapse, and possibly inherent differences in various strains of the same species. Observations with a second strain of *P. vivax* (Chesson) reveal that it has a much greater resistance to quinine than the McCoy strain.

Important features of the standard therapeutic test which require some comment are the control exercised over the variable of natural and acquired immunity, the duration of therapy, and selection of the time for reinoculation. In order to exclude immunity as an important determining factor in the experimental results, several measures were taken in the design of the routine testing procedure: (1) observations were restricted to individuals presumed to be completely susceptible; (2) treatment with the test drug was begun early in the course of the disease, *i.e.*, within five days after the onset of fever; and (3) a reinoculation procedure was utilized to test for continuing susceptibility in those persons who showed an apparently permanent therapeutic effect.

A stable plasma concentration of the test drug is maintained for four days in order that the period of therapy may encompass two complete cycles of schizogony. Although four days of therapy may be insufficient to interrupt permanently blood-induced infections due to all strains of *P. vivax*, it permits a wide gradation of thera-

peutic response and provides a basis for the comparative assay of other agents.

Selection of the time for reinoculation was based on the observation that, in blood-induced McCoy vivax, the spontaneous reappearance of parasites almost invariably occurs within 14 days after the last effective plasma drug level, if such a recrudescence is to be expected. The duration of the observation period which is necessary to separate Class II from Class III effects varies from strain to strain in any species of plasmodium as well as from species to species. With drugs that persist in the body, the interval between the termination of therapy and reinoculation must be extended to 14 days after the plasma drug concentration has reached a level known to have no therapeutic effect.

SUMMARY

1. A standard procedure suitable for the quantitative appraisal of drug activity in blood-induced vivax malaria has been described. The close relationship between plasma quinine concentration and therapeutic response makes possible the quantitative appraisal of drug activity with a limited number of experimental subjects.

2. By means of this procedure, it has been possible to demonstrate that the quinine-susceptibility of the erythrocytic phase of vivax malaria is a stable strain characteristic and that it is independent of the mode by which the malaria is transmitted.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

II. METHOD FOR THE QUANTITATIVE ASSAY OF SUPPRESSIVE ANTIMALARIAL ACTION IN FALCIPARUM MALARIA^{1, 2, 3}

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INTRODUCTION

It has been demonstrated in vivax malaria that the therapeutic response to quinine is closely related to the mean plasma quinine concentration achieved during a standard testing procedure (1). In addition, the susceptibility of the erythrocytic phase to quinine has been shown to be a stable characteristic of the McCoy strain of *P. vivax* and to differ quantitatively from that of the Chesson strain.

The human malarias due to the two other common species of plasmodium differ from vivax malaria in their biological and clinical characteristics and in their responses to chemotherapeutic agents. Therefore, it seemed advisable to determine their susceptibilities to quinine as a basis for the comparative assay of other antimalarials. However, infections due to *P. malariae* proved to be impractical for therapeutic testing because of their long incubation periods, the low density of peripheral

parasitemia, and the somewhat erratic course of the disease. Systematic studies were discontinued in view of these difficulties, together with the lesser importance of quartan malaria.

The present paper is concerned with studies of the susceptibility to quinine of the erythrocytic phase of two strains of falciparum malaria. All patients used in this study were neuro-syphilitics who presented no medical contraindication to therapeutic malaria. In general, the patients were those whose racial extraction or history of previous malaria made them unsuitable as experimental subjects in the vivax studies.

BLOOD-INDUCED MCCLENDON FALCIPARUM MALARIA

Falciparum (McClendon) ⁶ malaria, induced by an intravenous inoculum of 500,000 erythrocytic parasites, is characterized by a prepatent period of from two to 12 days (average five days). This is followed by an irregular, sustained fever and the rapid development of peripheral parasitemia which frequently reaches 50,000 per cu. mm. or higher by the second or third day after the first appearance of parasites in thick film preparations. In general, the onset of fever coincides with the first appearance of parasites in thick blood films. Only rarely does a patient achieve a parasite count as high as 10,000 per cu. mm. without fever during a primary attack.

Because excessive parasitemia and prolonged fever constitute a hazard to life in falciparum malaria, no deliberate attempt was made to observe

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A portion of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proceedings, 1946, 5, 216.

³ The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, and Wyeth, Inc., for contributing toward the publication costs.

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⁶ The McClendon strain of *P. falciparum* utilized in these studies was obtained from Doctor Martin D. Young.

the natural course of the disease. However, the invasive nature of McClendon falciparum and its potential virulence were attested by the facts that parasites in excess of 500,000 per cu. mm. were not uncommon, in spite of the early administration of antimalarial therapy, and that one patient died with what was presumed to be cerebral malaria.

Therapeutic interruption early in the course of the infection, as with McCoy vivax, may modify a second exposure to the disease. The onset of fever during recrudescence or following reinoculation usually coincides with a higher parasite density than is the case with the primary attack. The development of tolerance to the parasite in secondary or reinoculation infections may be interpreted as an indication of immunity acquired during the primary attack.

Among 285 individuals who were inoculated with McClendon falciparum, only two failed to develop a primary attack of malaria. Furthermore, the immunity which developed during the initial attack or during the subsequent observation period was in no case sufficient to interfere with either the anticipated clinical recrudescence or a prompt development of a clinical attack following reinoculation. This is presumably attributable to the early stage of the infection at which therapy was administered.

Routine of therapeutic tests

Each subject was inoculated with 500,000 parasites from an untreated patient in the first or second day of fever. Thick blood films were examined daily throughout the period of observation and parasite counts were obtained when indicated.

Therapy was started on the first or second day of fever over 101° F., or when parasitemia reached 50,000 per cu. mm. in the absence of fever. Quinine was administered with dosage regimens designed to maintain a stable plasma concentration for a standard number of days. Each course of therapy was initiated by a priming dose and continued with smaller doses at four- to six-hour intervals. All doses are recorded in terms of quinine base. Blood samples for the estimation of the plasma quinine concentration were obtained at sufficiently short intervals to permit an appraisal of the mean concentration each day. The mean plasma drug concentrations referred to throughout this paper are the averages of the individual daily mean concentrations.

The observation period subsequent to therapy began on the day following the last effective plasma drug level. In the event of the complete disappearance of parasites

and fever, the follow-up period was extended to at least 21 days, this interval being necessary to include the majority of renewals of clinical activity due to persistence of an erythrocytic phase. If, at the end of this period, there had been no evidence of renewed activity, reinoculation with one million parasites was performed and the patient followed until the recurrence of parasitemia. Therapeutic results were classified in three groups, as previously described for vivax infections (1).

The susceptibility of blood-induced McClendon falciparum malaria to quinine

The first series of observations involved the administration of quinine to 15 patients with blood-induced falciparum (McClendon) malaria (Table I). Stable plasma quinine concentrations were maintained for four days. Mean plasma quinine concentrations ranged from 2.1 to 10.4 mg. per liter. However, dosage regimens close to the upper limit of tolerance, when restricted to four days, result only in temporary therapeutic effects (Class II).

Therefore, therapeutic tests were performed in an additional 13 subjects in whom the period of effective plasma quinine concentration was extended to six days (Table II). Mean plasma drug concentrations ranged from 2.9 to 8.7 mg. per liter. Mean plasma quinine concentrations of 5.6 mg. per liter or higher, maintained for six days, consistently produced a permanent disappearance

TABLE I

The relationship between dosage and plasma concentration of quinine and therapeutic effect in four-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
You	1.50	10.4		x	
Jac	0.72	9.0		x	
Mor	1.50	8.3		x	
Ray	0.72	8.1			x
Ber	0.72	5.9		x	
Val	0.30	4.8		x	
Jun	0.15	4.7		x	
Jup	0.20	4.2		x	
Mor	0.30	3.6		x	
Yow	0.15	3.6		x	
Whi	0.15	3.5		x	
Gar	0.10	3.3		x	
Lun	0.20	3.2		x	
Sew	0.15	2.6	x		
Hop	0.15	2.1		x	

TABLE II

The relationship between dosage and plasma concentration of quinine and therapeutic effect in six-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Mus	0.36	8.7			x
Asb	0.70-1.5	7.6			x
Rag	0.36-0.70	7.3			x
Mit	1.00-1.50	6.8			x
Lee	0.36-0.55	5.9			x
McC	1.00-2.10	5.8			x
Car	0.55	5.6			x
Cha	0.30	5.4		x	
Gri	0.50-0.80	5.0		x	
Gra	0.46	4.5			x
Wil	0.30	3.2		x	
Har	0.30	3.1		x	
Gre	0.30	2.9		x	

of parasites and fever (Class III). Plasma quinine concentrations between 2.9 and 5.4 mg. per liter resulted in five Class II effects and one Class III.

Further evidence of the importance of the duration of therapy in evaluating the quinine susceptibility of the erythrocytic phase of McClendon falciparum was obtained by a study of six patients in whom plasma quinine concentrations were maintained for eight days (Table III). Here, the mean concentrations ranged from 3.2 mg. per liter to 7.2 mg. per liter. In all but one, a permanent interruption of the disease was obtained. In spite of the limited number of therapeutic tests performed, it is apparent that the therapeutic

TABLE III

The relationship between dosage and plasma concentration of quinine and therapeutic effect in eight-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Yat	0.72	7.2			x
Tai	0.72	6.1			x
Pco	0.72	4.1			x
Tho	0.72	4.1			x
Pow	0.72	4.1		x	
Ban	0.72	3.2			x

response to quinine in McClendon falciparum malaria is a function of the duration of therapy as well as the plasma quinine concentration.

BLOOD-INDUCED COSTA FALCIPARUM MALARIA

The variation in quinine-susceptibility between different strains of vivax malaria (1) prompted an examination of the antimalarial effect of quinine against another strain of *P. falciparum*.⁷ Sixteen patients were inoculated with Costa falciparum. The course of fever and parasitemia of Costa falciparum malaria is similar to that of the McClendon strain. In general, neither excessive parasitemia nor prolonged fever occurred as frequently in Costa falciparum infections as in McClendon malaria. Except for the strain of parasite used, the therapeutic testing procedure was the same as that previously described (1). Plasma quinine concentrations in the first group were maintained for six days.

Examination of the data in Table IV reveals that quinine administered for six days in nearly maximum tolerated doses does not generally produce a permanent interruption of the disease (Class III effect). There is no consistent re-

TABLE IV

The relationship between dosage and plasma concentration of quinine and therapeutic effect in six-day tests against blood-induced Costa falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Hun	2.10	14.8		x	
Hen	2.40	13.5		x	
Riv	1.55	12.6			x
McN	2.40	12.0		x	
Ton	1.55	11.6		x	
Bon	1.55	11.1		x	
Gre	1.00	10.5			x
Ell	2.40	9.9		x	
Moo	1.55	8.2		x	
Alv	0.77	8.2		x	
McN	1.55	7.3			x
Smi	0.77	7.0			x
Pug	0.40	5.1		x	
Bat	0.40	4.4		x	
Hen	0.40	4.1		x	
Mor	0.40	3.8		x	

⁷ The Costa strain was kindly furnished by Doctor Robert B. Watson. The strain was originally isolated by Doctor Mark F. Boyd.

TABLE V

The relationship between dosage and plasma concentration of quinine and therapeutic effect in eight-day tests against blood-induced Costa falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Car	1.55	12.0			x
Chu	0.77	7.7		x	
Mar	0.77	6.5		x	
Lug	0.77	5.4			x
Ham	0.77	2.8	x		

relationship between plasma drug concentration and therapeutic effect. An exploratory study of five patients in whom plasma quinine concentrations were maintained for a period of eight days (Table V) presents similarly inconsistent results. It may be concluded that the resistance of the Costa strain to the suppressive antimalarial action of quinine is greater than that of the McClendon strain.

MOSQUITO-INDUCED COSTA FALCIPARUM MALARIA

Three patients were infected by the bites of *A. quadrimaculatus* mosquitoes whose salivary glands contained sporozoites of the Costa strain of *P. falciparum*. In other respects, the procedure of the therapeutic test was the same as that previously described for mosquito-induced vivax malaria (1).

The data are limited, but the results (Table VI) indicate a high degree of quinine resistance of the erythrocytic phase of the mosquito-induced dis-

TABLE VI

The relationship between dosage and plasma concentration of quinine and therapeutic effect in eight-day tests against mosquito-induced Costa falciparum malaria

Patient	Daily dose	Mean plasma quinine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Kir	1.6	13.0		x	
Kih	1.6	10.2		x	
Cop	1.6	8.3			x

ease, similar to that observed with the same strain when induced by the inoculation of infected blood.

DISCUSSION

A standard testing procedure, modified in certain respects from that utilized for McCoy vivax malaria (1), was employed in determining the quinine-susceptibility of the McClendon strain of *P. falciparum*. This strain has a greater resistance to the suppressive antimalarial action of quinine than the McCoy strain of *P. vivax*, in terms of the plasma quinine concentration, oral dosage, or duration of therapy required to produce a given therapeutic effect. The difference between the McClendon strain of *P. falciparum* and the Chesson strain of *P. vivax* is less marked. Within the falciparum species, it has been demonstrated that the quinine-susceptibility of the erythrocytic phase differs significantly in different strains of the parasite. This evidence constitutes a quantitative expression of the clinical experience of many observers of falciparum malaria in endemic areas (2).

It is pertinent to note that resistance to the action of quinine is not necessarily related to the severity or virulence of the clinical disease. Costa falciparum, although more resistant to quinine than the McClendon strain, is nevertheless far less hazardous to life.

It is not intended that the appraisal of drug activity in blood-induced falciparum should replace studies utilizing vivax malaria. The two procedures are complementary in that they yield information on the comparative effectiveness of an antimalarial agent in both types of infection. It should be appreciated that, due to an apparent lack of a persisting tissue phase in falciparum malaria, the termination of the erythrocytic phase is tantamount to a cure.

CONCLUSIONS

1. The therapeutic response of blood-induced McClendon falciparum malaria to quinine has been shown to be related to the plasma quinine concentration and the duration of therapy, both of which factors are amenable to quantitative definition.

2. Therefore, blood-induced malaria due to this

strain should provide a suitable test object for the quantitative appraisal of the relative suppressive activities of antimalarial agents.

3. As in vivax malaria, the quinine-susceptibility of the erythrocytic phase differs in various strains of *P. falciparum*. Costa falciparum infections are more resistant to quinine than are those due to the McClendon strain.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

III. THE PHYSIOLOGICAL DISPOSITION AND ANTIMALARIAL ACTIVITY OF THE CINCHONA ALKALOIDS^{1, 2, 3}

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INTRODUCTION

The relative effectiveness of the various cinchona alkaloids in the treatment of malaria has been the subject of numerous investigations since the meeting of the Madras Commission in 1868 (1). Without exception, studies undertaken prior to the recent war involved the appraisal of the comparative antimalarial activities of quinine, quinidine, cinchonine and cinchonidine on the basis of the oral dosage administered during one or another standard therapeutic regimen. Although the conclusions derived from these studies are not in complete agreement, it has been the general impression that all four of the cinchona alkaloids possess roughly equivalent antimalarial activities.

Therefore, it is surprising that quinine alone has come into general use in the suppression and treatment of malaria. The need for a further appraisal of the other cinchona alkaloids arose early in the course of the war because of the shortage of effective antimalarial drugs. Various cinchona barks containing the principal crystallizable alka-

loids other than quinine were potentially available in the Western hemisphere and offered a partial solution to the acute shortage of antimalarial agents.

The studies reported in this communication are concerned with the physiological disposition, antimalarial activity and, to a lesser extent, the toxicity of the four principal cinchona alkaloids. The recent development of simple, reliable methods for estimating the concentrations of the alkaloids in biological materials permitted an examination of certain aspects of their pharmacology and therapeutic activity in terms of measured drug concentrations. Quantitative information of this type may be expected to establish a basis for the most effective use of these drugs in the treatment of malaria.

MATERIALS AND METHODS

In the estimation of the concentrations of cinchona alkaloids in plasma several general procedures were utilized. The fluorescent intensity of quinine was measured in an acidic, aqueous medium following protein precipitation with metaphosphoric acid (2), quinidine by fluorimetry after extraction of the drug into ethylene dichloride (3). Cinchonine and cinchonidine were measured by the formation of an organic-soluble, water-insoluble complex of the alkaloid with methyl orange (4). With the exception of the precipitation method, the final procedures utilized in estimating plasma drug concentrations appear to be specific for the parent compounds. Ten to 20 per cent of the total fluorescent intensity obtained when the metaphosphoric acid precipitation method is applied to human plasma is contributed by metabolic products of quinine.

The therapeutic tests with blood-induced malaria were performed in accordance with standard procedures outlined in the preceding papers (5, 6). Therapy was started on the fourth day after the onset of fever in vivax malaria and on the first or second day of fever in falciparum malaria. Stable plasma drug concentrations were maintained during the standard four-day (vivax) or six-

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² Portions of this work were presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 206 and 216.

³ The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, and Wyeth, Inc., for contributing toward the publication costs.

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day (*falciparum*) therapeutic period. The drugs were administered in soft gelatine capsules. All doses and plasma drug concentrations are reported in terms of the free base. The therapeutic results are classified as previously defined: Class I, indicating no certain effect from drug administration; Class II, a temporary suppression of parasitemia and/or fever; and Class III, a "permanent" effect followed by a positive reinoculation procedure to establish evidence of continuing host-susceptibility.

PHYSIOLOGICAL DISPOSITION

The studies reported in this section were directed toward a limited description of the factors entering into the physiological disposition of the cinchona alkaloids. Particular emphasis was placed on the relationship between oral dosage regimens and the resultant plasma drug concentrations. In addition, data are presented concerning the absorption of the alkaloids from the gastrointestinal tract, their distribution in the body, metabolic alteration, and excretion.

Each of the drugs was administered according to several dosage schedules for periods of four days or more. Therapy consisted of an initial priming dose, usually one-half of the total daily dose, followed by small maintenance doses at four- to eight-hour intervals. Blood samples for the estimation of plasma drug concentrations were obtained several times daily throughout the period of therapy. The mean plasma drug concentrations

referred to in this paper are the averages of the individual daily mean concentrations. The various dosage schedules and the resultant plasma concentrations of each drug are summarized in Table I.

A wide range of mean plasma drug concentrations is obtained in different individuals receiving the same oral dose of any one of the four alkaloids. However, it may be noted that quinine produces the highest plasma drug concentrations. The most striking difference is that between quinine and cinchonine. At total daily doses of approximately 1.0 gram, plasma cinchonine concentrations are generally less than 5 per cent of those of quinine. Quinidine and cinchonidine concentrations are intermediate to those of cinchonine and quinine. Note should be taken of a second important difference between cinchonine and the other three alkaloids. With increasing doses of cinchonine, the resultant plasma concentrations are increased out of proportion to the increments of dosage. With the other alkaloids, the increases are disproportionately small in relation to the dose.

Peak plasma drug concentrations are achieved within one to three hours after a single oral dose of any one of the four drugs. Single oral doses of the alkaloid frequently produce plasma drug concentrations considerably lower than those obtained with soluble salts. However, on maintenance regimens, the equilibrium plasma concentration is the same with either alkaloid or salt. The plasma drug concentrations fall fairly rapidly after the termination of therapy, so that only negligible quantities of the drugs persist in the plasma beyond 24 hours.

The absorption of the four drugs from the gastrointestinal tract is essentially complete, insofar as less than 5 per cent of the serially administered doses can be recovered from the stools. Nor is there evidence that appreciable metabolic alteration of the alkaloids occurs during their passage through the digestive tract. Small amounts of cinchonine may be incubated with stool at 37° C. and recovered quantitatively. That absorption from the gastro-intestinal tract is rapid and essentially complete is confirmed by the observation that plasma quinine and cinchonine concentrations obtained one to four hours after a single intravenous dose are approximately the same as when the drugs are administered by the oral route.

TABLE I

Dosage schedules of cinchona alkaloid and resultant plasma drug concentrations

Drug	Total daily dose	Number of subjects	Mean plasma drug concentration	
			Range	Average
Quinine	grams (base)		mg./L	mg./L
	0.20	20	1.9-5.1	3.3
	0.50	6	2.9-6.5	4.9
	0.75	9	5.5-9.5	6.3
Cinchonine	1.50	44	7.0-12.9	9.6
	0.50	6	<0.05	<0.05
	1.00	13	0.1-0.5	0.2
	2.00	16	0.3-2.7	1.0
Quinidine	0.1	10	0.2-1.3	0.7
	0.6	4	1.4-3.3	2.0
	1.5	3	2.6-5.0	3.5
Cinchonidine	0.3	6	0.8-3.2	1.7
	1.0	13	1.2-3.8	2.2
	2.0	6	2.3-9.2	5.5

TABLE II
Distribution of quinine in human blood

Patient	Quinine concentration		Cell quinine concentration, per cent of plasma quinine concentration
	Plasma	Cells	
	mg./L	mg./L	
1	5.32	0.94	18
2	3.97	0.78	19
3	5.36	0.96	17
4	8.24	1.05	13
5	3.69	0.65	18

Each of the subjects received a single oral dose of 0.3 gram quinine hydrochloride. A specimen of blood was obtained two hours after the dose, and the plasma was promptly removed by centrifugation.

Few direct observations on the extent to which the alkaloids are localized in tissues have been possible in man. The distribution of quinine in blood was examined in five subjects two hours after a single dose of 0.3 gram (Table II). The concentration of quinine in the cellular elements of the blood was found to be less than 20 per cent of that in the plasma. Similar results were obtained with cinchonine. Thus, it is apparent that little localization of quinine or cinchonine occurs in either erythrocytes or leucocytes under the conditions of this experiment. Distribution studies in the chicken and dog (7, 8) have shown that the alkaloid concentrations in liver, spleen and other parenchymatous tissues are approximately 10 to 30 times that in the plasma. A lesser degree of localization occurs in skeletal and cardiac muscle, brain and other tissues. In man, the apparent volume of distribution of quinine within 15 minutes after an intravenous dose approximates the body weight. This may be regarded as indirect evidence for the limited tissue localization of quinine.

The total urinary excretion of unaltered cinchona alkaloids amounts to less than 5 per cent of the administered drug. Various metabolic products of the alkaloids are recovered from the urine and account for an additional 65 per cent of the total dose in the case of cinchonine, 20 per cent of quinine, but less than 5 per cent of either quinidine or cinchonidine (9, 10). The fractions unaccounted for may represent metabolic products not readily identified. However, it is evident that renal excretion plays a minor role in regulating

the plasma concentrations of the parent compounds.

In view of the essentially complete absorption, limited localization and limited excretion of the cinchona alkaloids, it becomes apparent that the fate of these drugs in the body is primarily one of metabolic alteration. As noted above, varying proportions of orally administered doses can be accounted for in the urine in the form of various metabolic products. Certain important metabolic products have been recovered from urine by selective extraction into organic solvents and isolated by means of the counter-current distribution technique (10). Tentative identification by elementary analysis and comparison with known compounds indicates that each of the four alkaloids is oxidized to the corresponding carbostyryl (2-hydroxyquinoline). In some instances, oxygen appears to be added to the quinuclidine portion of the molecule (9). An enzyme which oxidizes each of the four alkaloids to its corresponding carbostyryl has been isolated from rabbit liver (11). This enzyme has properties similar to, and is not dissociable from the flavoprotein liver aldehyde oxidase. However, at the present time there is no evidence that this enzyme is responsible for the oxidation of the cinchona alkaloids in man. Moreover, it appears probable that the exact metabolic pathway differs somewhat with the various alkaloids. The plasma levels of the various alkaloids achieved on maintenance dosage schedules reflect their relative rates of metabolic alteration (Table I). Consequently, the low plasma cinchonine levels obtained in man reflect a high rate of metabolic alteration rather than extensive tissue localization.

ANTIMALARIAL ACTIVITY

Each of the four cinchona alkaloids was tested for its ability to terminate acute attacks of blood-induced malaria. The results obtained with quinine have been described in detail in the preceding papers. In McCoy vivax malaria, a mean plasma quinine concentration of 5 mg. per liter or higher, maintained for four days, permanently interrupts the erythrocytic asexual phase of the infection. Quinine concentrations between 2 and 5 mg. per liter produce a temporary suppression of parasitemia and fever, while concentrations of

TABLE III

The relationship between dosage and plasma concentration of cinchonine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

Patient	Daily dose	Mean plasma cinchonine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Str	2.00	0.9			x
Bis	2.00	0.8			x
Sjo	2.00	0.5			x
Spa	1.00	0.5			x
Erl	2.00	0.4			x
Kol	1.00	0.4			x
Fre	0.80	0.3			x
Car	0.75	0.3			x
Tho	0.80	0.1			x
Joh	1.00	0.1			x
Gri	0.80	0.1			x
Mar	1.00	0.1			x
Dai	0.50	0.07		x	
Tul	0.50	<0.05		x	
Ant	0.50	<0.05		x	
Coh	0.50			x	
Rei	0.50			x	
Nim	0.50			x	

less than 2 mg. per liter have no effect on the course of the disease. Approximately the same plasma quinine concentrations must be maintained for six days to produce comparable effects in malaria due to the McClendon strain of falciparum. In addition, it was demonstrated that the susceptibility of the erythrocytic phase of malaria due to a particular strain of plasmodia is independent of the mode of transmission, i.e., blood-induced or mosquito-induced. For this reason, the present studies were confined to blood-induced infections.

Table III summarizes the results of therapeutic tests with cinchonine in 18 subjects with McCoy vivax malaria. Class III effects were obtained in the 12 individuals with mean plasma cinchonine concentrations of 0.1 mg. per liter or higher. Concentrations of less than 0.1 mg. per liter were consistently associated with Class II effects. Cinchonine was administered for six days to 15 patients with McClendon falciparum malaria (Table IV). In this infection, mean plasma cinchonine concentrations of 1.0 mg. per liter or higher consistently produced Class III effects. Of six individuals with mean concentrations of 0.5 or 0.6 mg. per liter, four showed Class III effects, and two showed Class II effects. No effect could be observed in one individual with a

TABLE IV

The relationship between dosage and plasma concentration of cinchonine and therapeutic effect in six-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma cinchonine concentration	Class of therapeutic effect		
			I	II	III
	grams (base)	mg./L			
Vel	2.0	2.7			x
Nad	2.0	2.0			x
Gar	2.0	2.0			x
Jef	2.0	1.9			x
Cra	2.0	1.6			x
Car	2.0	1.0			x
Gor	1.5	0.6			x
Abb	2.0	0.6			x
Boo	1.5	0.6		x	
Lok	2.0	0.5		x	
Naz	1.5	0.5			x
Cue	1.5	0.5		x	
Vas	1.0	0.2		x	
Moc	1.0	<0.1		x	
Dil	1.0	<0.1	x		

mean concentration of less than 0.1 mg. per liter.

Quinidine was studied in nine patients with McCoy vivax malaria (Table V) and in 19 patients with McClendon falciparum malaria (Table VI). In both infections, the critical plasma quinidine concentration separating Class III and Class II effects was found to be approximately 1.0 mg. per liter.

Cinchonidine was studied in 11 patients with McCoy vivax malaria (Table VII) and in eight patients with McClendon falciparum malaria (Table VIII). The critical plasma cinchonidine concentration separating Class III and Class II

TABLE V

The relationship between dosage and plasma concentration of quinidine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

Patient	Daily dose	Mean plasma quinidine concentration	Class of therapeutic effect	
			II	III
	grams (base)	mg./L		
Tur	.24	3.2		x
Swe	.24	2.2		x
Gar	.24	1.9		x
Fein	.1	1.3		x
Heg	.24	0.9		x
McL	.1	0.8	x	
Rom	.1	0.7	x	
She	.1	0.5	x	
Ped	.075	0.4	x	

TABLE VI

The relationship between dosage and plasma concentration of quinidine and therapeutic effect in six-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma quinidine concentration	Class of therapeutic effect		
			I	II	III
	<i>grams (base)</i>	<i>mg./L</i>			
All	1.0	5.0			x
Win	1.0	3.5			x
For	0.75	3.3			x
Jac	0.5	3.3			x
McC	1.0	3.0			x
Sep	0.5	2.6			x
LaV	0.6	1.9			x
Wri	0.2	1.8			x
Dia	0.4	1.6			x
Bra	0.4	1.4			x
McC	0.3	1.1			x
Wil	0.15	1.0		x	
Agr	0.10	1.0		x	
Gra	0.7	0.8			x
Bow	0.1	0.8		x	
Cib	0.1	0.7		x	
Hud	0.1	0.6		x	
San	0.1	0.6		x	
Sei	0.1	0.2	x		

effects in both infections was between 2.0 and 3.0 mg. per liter.

It is apparent that the plasma drug concentration which permanently interrupts the erythrocytic phase of either vivax or falciparum malaria differs significantly with the various alkaloids. Effective plasma concentrations of cinchonine are one-tenth to one-fiftieth those of quinidine. Quinidine and cinchonidine are effective in concentra-

TABLE VII

The relationship between dosage and plasma concentration of cinchonidine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

Patient	Daily dose	Mean plasma cinchonidine concentration	Class of therapeutic effect	
			II	III
	<i>grams (base)</i>	<i>mg./L</i>		
Bur	2.0	7.3		x
Dai	1.0	3.3		x
Mur	0.6	3.2		x
Ode	0.36	3.0		x
Pow	0.6	2.9		x
Jac	0.6	2.9		x
Mul	0.78	2.0	x	
Saw	0.6	1.7		x
Cur	0.36	1.5	x	
Rip	0.36	1.2	x	
Oli	0.36	1.0	x	

TABLE VIII

The relationship between dosage and plasma concentration of cinchonidine and therapeutic effect in six-day tests against blood-induced McClendon falciparum malaria

Patient	Daily dose	Mean plasma cinchonidine concentration	Class of therapeutic effect	
			II	III
	<i>grams (base)</i>	<i>mg./L</i>		
Smi	1.0	5.2		x
Har	1.0	3.1		x
Cha	0.6	2.8		x
Nap	0.6	2.4		x
Joh	0.6	2.0	x	
Cho	0.6	1.9	x	
Smi	0.6	1.8	x	
Jim	0.4	1.3	x	

tions one-fifth and one-half, respectively, those of quinine. However, a final appraisal of the comparative effectiveness of the various alkaloids requires some consideration of the dosage regimens which yield the effective plasma drug concentrations. It was noted in the preceding section that a given dosage regimen results in very different plasma concentrations of the various alkaloids. Therefore, Table IX summarizes the minimal plasma drug concentrations required for Class III effects in McCoy vivax malaria and the approximate daily doses of the alkaloids which yield these critical concentrations. On this basis of comparison, it may be concluded that the antimalarial activities of the four alkaloids are of the same order of magnitude. Quinidine is approximately twice as active as quinine, while cinchonine and cinchonidine are about one-half as active as quinine. A striking deviation from these generalizations is the relatively greater resistance of McClendon falciparum to cinchonine therapy. However, these findings provide a general confirmation of numerous previous studies.

The extremely low plasma concentrations of cinchonine which are effective in vivax malaria prompted an examination of the antimalarial activity of cinchonine carbostyryl, the principal metabolic product present in plasma and recoverable from urine. Therapeutic tests were performed in five patients with McCoy vivax malaria (12). One Class III effect was obtained at a mean plasma carbostyryl concentration of 3.4 mg. per liter. Class II effects occurred at concentrations of 2.0 and 1.3 mg. per liter, and no effect was

TABLE IX

Relative activity of the four major cinchona alkaloids

Alkaloid	Mean plasma concentration for class III	Approximate daily dose	Quinine equivalent
		grams (base)	
Quinine	5.0	0.50	1
Quinidine	1.0	0.25	2
Cinchonine	0.1	1.00	$\frac{1}{4}$
Cinchonidine	2.5	1.00	$\frac{1}{4}$

demonstrable in two patients with concentrations of 1.1 and 0.7 mg. per liter. A daily oral dose of 3.0 grams of the carbostyryl was required to produce the Class III effect. The suppressive antimalarial activity of the carbostyryl is, therefore, much less than that of cinchonine itself. Since, dose for dose, the same plasma carbostyryl concentration is obtained whether cinchonine or its carbostyryl is administered (12), it appears that this metabolic product contributes little to the antimalarial effect obtained with cinchonine.

TOXICITY

No attempt was made to appraise the relative toxicities of the various cinchona alkaloids in man. However, several incidental observations during studies of physiological disposition and antimalarial activity are worthy of mention. Typical cinchonism occurred frequently during the routine administration of quinine. Tinnitus and impairment of hearing rarely occurred at plasma quinine concentrations of less than 10 mg. per liter. Approximately half of the individuals receiving total daily doses of 1.5 grams of quinine complained of these symptoms. Frank idiosyncrasy to quinine was noted in only one of several hundred patients receiving quinine. The hypersensitivity was manifested by extreme flushing of the skin and intense generalized pruritus following small doses administered either orally or by intravenous injection.

The majority of patients receiving 3.0 to 4.0 grams of cinchonine daily experienced blurring of vision, dizziness, drowsiness, marked dryness of the mouth, and constipation. In addition, acute urinary retention occurred in several instances and necessitated catheterization. Daily doses of

2.0 grams or less produced none of these unpleasant side-effects.

No evidences of toxicity were encountered at total daily doses of 2.0 grams of cinchonidine or 1.0 gram of quinidine.

DISCUSSION AND SUMMARY

It is evident from the data presented that any one of the four cinchona alkaloids will be generally effective in the management of clinical attacks of malaria. Totaquine, U.S.P., and other similar preparations, are cheap and available in abundance. These contain varying proportions of each of the cinchona alkaloids and should be useful antimalarials unless the activities of the alkaloids are not additive. There is definite evidence that the effect of combinations of the different cinchona alkaloids is additive, both in the avian malaras (13), and *in vitro* against falciparum parasites (14). Totaquine has been utilized rather extensively in many endemic regions and has been reported to be effective. However, until recently, there was no definitive assay of the toxicity and antimalarial activity of this type of preparation (15).

The data also indicate the importance of the various factors which determine the overall physiological disposition of each cinchona alkaloid. The cinchona alkaloids are localized in the tissues to some extent, but not nearly as much as is quinacrine (16) and the 4-aminoquinolines (17), and there is no accumulation of the drugs in the body. The combined rates of metabolism and excretion of the alkaloids are such that dosage every six hours is required to assure the maintenance of effective plasma drug levels. Consequently, it would be unlikely that the cinchona alkaloids could be as effective or as simply administered for suppressive purposes, as can some of the newer synthetic antimalarial agents which can be given at weekly intervals.

The effective plasma level of cinchonine is considerably lower than that for any of the other cinchona alkaloids. However, its metabolism proceeds at such a rapid rate that plasma drug levels achieved on cinchonine dosage are also lower than those of the other alkaloids. Since the first metabolic product of cinchonine is its carbostyryl, it was possible that blocking the 2 position might re-

sult in a compound that would be metabolized at a slower rate and which, therefore, might be a more effective antimalarial agent. This was not attempted with cinchonine, but the line of reasoning led to the development of a new series of synthetic compounds that were found to be active antimalarial agents. These compounds, 2-phenyl quinoline methanol derivatives, are readily synthesized but do not possess the advantages inherent in quinacrine and the 4-aminoquinolines (18).

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

IV. THE METABOLISM OF CINCHONINE IN RELATION TO ITS ANTIMALARIAL ACTIVITY^{1, 2, 3}

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The study of the host metabolism of a chemical agent has both theoretical and practical interest in any consideration of chemotherapeutic action. Metabolic change can modify chemotherapeutic effect through a conversion of an active to an inactive agent or through a conversion of an inactive to an active agent. Or activity itself may in some way be associated with the process of metabolism. The cinchona alkaloids were selected for special study since it appeared that they offered a good opportunity to obtain information which might bear on these generalizations. The cinchona alkaloids are extensively metabolized by the tissues of man and certain animals (1, 2, 3), their fate being primarily determined by the rate of their metabolic alteration (4).

A number of metabolic products formed in man from quinine, quinidine, cinchonine and cinchonidine have been isolated (3) and some, characterized (3, 5). When administered to human subjects, cinchonine is metabolized much more rapidly than any of the other cinchona alkaloids, with

the production of large amounts of 2-hydroxy cinchonine (cinchonine carbostyryl), and smaller amounts of a further oxidation product. The latter appears to be a derivative of 2-hydroxy cinchonine with an additional oxygen in the quinclidine portion of the molecule (3, 5).

MATERIALS AND METHODS⁵

With the exception of a few studies on patients with active vivax malaria, the data on the physiological disposition of cinchonine and 2-hydroxy cinchonine were collected from experiments performed in normal volunteers.

The per cent of various amounts of cinchonine and its 2-hydroxy derivative bound on plasma albumin was determined by a technique previously described (6). Absorption from the gastro-intestinal tract was examined by determining the amount of drug excreted with the feces per 24 hours while the subjects were receiving serial oral doses. This procedure yields valid figures since neither cinchonine nor the 2-hydroxy compound was destroyed when incubated at 37° C. for 24 hours in a mixture of feces. Renal excretion was determined through the use of 24-hour collections of urine while subjects were receiving cinchonine or its 2-hydroxy derivative. The completeness of urine collections was checked by serial determination of creatinine excretions. The renal clearances of cinchonine and 2-hydroxy cinchonine were determined over periods of one hour. Forty-five minutes prior to each clearance, the subject was given 500 ml. of water by mouth. The relation between oral dosage of cinchonine and its 2-hydroxy derivative and the resulting plasma drug levels was determined from the average plasma drug concentrations of the last three of four or five days on serial dosage. Blood samples were obtained three hours after the first daily dose.

The antimalarial activity of 2-hydroxy cinchonine was examined in five patients with blood-induced McCoy strain vivax malaria in accordance with standard procedure previously outlined (7). Briefly, 2-hydroxy cinchonine was administered by dosage schedules which produce

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² Portions of this work were presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 175 and 214, and were also discussed in a Harvey Lecture by Dr. James A. Shannon, Oct. 25, 1945. Permission to use Table VI has been obtained from the Harvey Society.

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⁵ The 2-hydroxy cinchonine utilized in these studies was isolated by Dr. B. B. Brodie and J. E. Baer from the urine of normal human volunteers who were receiving large oral doses of cinchonine.

fairly stable plasma drug concentrations during the four-day therapeutic period. The therapeutic results are classified in three groups: Class I, no certain effect; Class II, a temporary suppression of parasitemia and/or fever; and Class III, a "permanent" effect, *i.e.*, an absence of parasitemia for 14 days, followed by a positive reinoculation to demonstrate continuing host-susceptibility to the infection.

Chemical methods^a

A. Cinchonine. The concentration of cinchonine in biological fluids is measured by a method that is specific in that metabolic products of cinchonine are excluded (8).

B. 2-Hydroxy-cinchonine. 2-hydroxy cinchonine is separated from the biological material by extraction into ether at pH 10. About 80 per cent of the compound is extracted by this procedure, the exact amount depending on the relative volumes of the two phases and on the temperature. The distribution coefficient, however, is not greatly affected by small changes in temperature. The ether phase is evaporated to dryness and the residue taken up in ethylene dichloride. The ethylene dichloride is shaken with methyl orange solution at pH 5. The methyl orange, which dissolves in the solvent through salt formation with the 2-hydroxy cinchonine, is measured photometrically.

The levels estimated by the method described for 2-hydroxy cinchonine must be corrected for cinchonine where it is present.

Reagents:

1. Standard 2-hydroxy cinchonine (10 mgm. per 100 ml.). Ten mgm. of the free base are dissolved in 100 ml. of 0.01 N H₂SO₄. Working standards are prepared by dilution of this solution with 0.01 N H₂SO₄. The standard solutions deteriorate slightly after three to four days and it is therefore advisable to make fresh standards at least twice a week.

2. Borate buffer—pH 10. To 50 ml. of 0.2 M boric acid in 0.2 M KCl add 43.9 ml. 0.2 N NaOH and dilute to 200 ml. with water.

3. 1 N NaOH.

4. Ether. A reagent grade of absolute ether is purified by washing with an equal volume of 1 N HCl followed by three washes with water.

5. Ethylene dichloride containing 1 per cent ethanol. A technical grade of ethylene dichloride is purified by successive washings with equal volumes of 1 N NaOH and 1 N HCl, followed by two washings with water. One per cent by volume of ethanol is added.

6. Alcoholic H₂SO₄. Two per cent (by volume) concentrated H₂SO₄ in alcohol.

7. Methyl orange solution. Dissolve 90 mgm. of the sodium salt of methyl orange in 100 ml. of 0.5 M boric acid solution by gentle heating. Cool the solution to

room temperature and filter if necessary. Wash the solution several times by shaking with an equal volume of ethylene dichloride.

Procedure:

To 5 ml. of plasma in a 60 ml. glass-stoppered bottle add 1 N NaOH until the pH is about 10 (test with hydron paper). This usually requires 0.1 ml. of NaOH. In the case of urine, add 4 ml. of pH 10 borate buffer to 1 ml. of urine, diluted if necessary. Then add 30 ml. of ether and shake for 15 minutes on a shaking apparatus. Allow the phases to separate, centrifuging the bottle if necessary. Transfer 20 ml. of the ether to another glass-stoppered bottle and evaporate to dryness on a water bath. Dissolve the residue in 10 ml. of alcoholic ethylene dichloride, add 0.5 ml. of methyl orange solution, and shake for five minutes. Transfer the contents to a 25 ml. test tube and centrifuge for ten minutes at 3000 r.p.m. Carefully remove all the supernatant layer by aspiration, decant the ethylene dichloride phase into another test tube, and recentrifuge for five minutes. Pipette 10 ml. of the ethylene dichloride into a colorimeter tube containing 2 ml. of the alcoholic H₂SO₄ and mix thoroughly. Read in the colorimeter at 540 mμ.

The reagent blank in which water is substituted for plasma is run through the same procedure and is used for setting the instrument to zero optical density.

Standard curve:

Working standards are prepared by taking 1 ml. of standard solution, adding 4 ml. of the borate buffer and 30 ml. of ether and handling in the same manner as for the plasma and urine determination. Standards are run concurrently with each set of determinations to compensate for variations in the distribution coefficient of 2-hydroxy cinchonine between water and ether at different temperatures.

Calculation of results:

A. When no cinchonine is present

$$\frac{R_u}{R_s} \times A_s \times \frac{1}{\text{ml. sample}}$$

= mgm. of 2-hydroxy cinchonine in 1 liter of fluid

where R_u = optical density unknown.

R_s = optical density standard.

A_s = micrograms of 2-hydroxy cinchonine in standard.

B. When cinchonine is present

$$\frac{R_u - KY}{R_s} \times A_s \times \frac{1}{5}$$

= mgm. of 2-hydroxy cinchonine in 1 liter of fluid

where R_u = optical density unknown.

R_s = optical density standard.

A_s = micrograms of 2-hydroxy cinchonine in standard.

K = optical density resulting from 1 μg. cinchonine in 5 ml. of 0.1 N H₂SO₄, alkalized and run through the 2-hydroxy cinchonine procedure.

Y = micrograms cinchonine per liter of sample, as determined by the benzene method.

^a The authors acknowledge with thanks the advice and assistance of Dr. B. B. Brodie in the development of the method for the measurement of 2-hydroxy cinchonine concentration.

Sensitivity and precision:

An optical density of about 0.200 is obtained on the Coleman Model 6 spectrophotometer when 20 μ g. of 2-hydroxy cinchonine is carried through the procedure. Known amounts of the compound added to plasma are recovered with an error of ± 5 per cent both in the presence and absence of cinchonine. There is a small plasma blank which, for 5 ml. plasma, ranges in optical density from .007 to .046 with a mean of .015. The blank for urine is negligible in relation to the large amount of 2-hydroxy cinchonine.

RESULTS

Gastro-intestinal absorption. Two to 3 per cent of administered cinchonine or its 2-hydroxy derivative can be recovered in the feces of subjects who receive 2 or 3 grams of either compound daily in serial oral dosage. This indicates almost complete absorption of both compounds.

Plasma protein binding. Both cinchonine and 2-hydroxy cinchonine are bound to a considerable extent on non-diffusible constituents of plasma, presumably the albumin fraction. At a plasma drug concentration of 2 mgm. per liter and a plasma albumin content of 4 grams per 100 ml., 60 per cent of cinchonine and 52 per cent of 2-hydroxy cinchonine are bound. These observations were made at 37° C. and a pH of 7.4. At lower plasma drug concentrations the per cent bound is greater, although accurate results could not be obtained because the methods of analysis were not sensitive enough.

Relation between serial oral dosage and plasma drug levels. Plasma drug levels achieved on serial oral dosage of cinchonine and its 2-hydroxy derivative are summarized in Table I. Considerably higher levels are achieved by 2-hydroxy cinchonine than by equal doses of cinchonine. This is especially striking at the lowest dose studied.

TABLE I

Relation between serial oral dosage of cinchonine (C) and 2-hydroxy cinchonine (CMP) and plasma drug levels
(Plasma drug levels represent average of last three of four to six days on drug)

Daily dose	Cinchonine		2-Hydroxy cinchonine	
	Number of subjects	Mean plasma C level	Number of subjects	Mean plasma CMP level
grams		mgm./L		mgm./L
0.5	10	0.06	2	0.9
2.0	10	0.6	2	1.8
3.0	10	1.5	1	3.4

TABLE II

Relation between serial oral dosage of cinchonine and plasma cinchonine (C) and 2-hydroxy cinchonine (CMP) levels
(Plasma drug levels represent average of last three of five days)

Daily dose	Plasma drug levels (mgm./L)							
	Subject 1		Subject 2		Subject 3		Average	
	C	CMP	C	CMP	C	CMP	C	CMP
grams								
0.5	0.04	0.65	0.05	1.22	0.07	0.70	0.05	0.86
1.0	0.30	0.88	0.31	1.43	0.33	1.11	0.31	1.14
2.0	0.62	2.47	1.03	3.48	0.83	3.33	0.83	3.09

When cinchonine is administered by mouth and the plasma analyzed for both cinchonine and 2-hydroxy cinchonine (Table II), the two compounds are found in the same relative concentrations as when the drugs are given separately. Compare the 0.5 and 2.0 gram doses in Tables I and II.

Renal excretion of cinchonine and 2-hydroxy cinchonine. The renal clearance of cinchonine and its 2-hydroxy derivative was examined in three subjects with normal kidneys. These data are summarized in Table III. The clearance of the 2-hydroxy compound is higher than that of cinchonine itself and approaches the rate of renal plasma flow. As has been found with other organic bases (9, 10), the clearance of cinchonine can be depressed by the concomitant administration of large doses of sodium bicarbonate. The clearance of 2-hydroxy cinchonine, however, is not significantly altered by this procedure.

The 24-hour excretion in urine of serially administered cinchonine both as cinchonine and as the 2-hydroxy derivative is shown in Table IV. On the average, only 3.6 per cent of the dose is recovered as cinchonine while 2-hydroxy cin-

TABLE III

Renal clearance of cinchonine (C) and 2-hydroxy cinchonine (CMP)
(On oral dosage cinchonine, same three subjects)

Group	Number of experiments	Average clearance, ml./min.	
		C	CMP
Without NaHCO ₃	5	193	521
With NaHCO ₃	8	93	(59)

TABLE IV

24-Hour excretion of cinchonine (C) and 2-hydroxy cinchonine (CMP) on serial oral dosage of cinchonine
(Excretion values represent average of last three of five days on each dose)

Daily dose	24 Hour excretion, milligrams									Average per cent daily dose recovered	
	Subject 1			Subject 2			Subject 3				
	Creatinine	C	CMP	Creatinine	C	CMP	Creatinine	C	CMP	C	CMP
grams											
0.5	1,980	11	319	2,020	11	306	1,740	10	243	2.0	57.8
1.0	2,010	35	584	1,870	39	468	1,740	44	367	3.9	47.3
2.0	1,700	124	1,070	1,600	82	1,200	1,710	83	1,260	4.8	60.5
							Average:			3.6	55.2

chonine accounts for 55 per cent of the dose. When 2-hydroxy cinchonine alone is administered in the same fashion, only 34 per cent is recovered as such (Table V). All the subjects shown in Table V had active vivax malaria at the time of the observations. In another single experiment only 29 per cent of a single oral dose of 2 grams 2-hydroxy cinchonine was recovered in the urine excreted by a normal subject during the subsequent 48 hours.

Antimalarial activity

2-hydroxy cinchonine exhibited a definite but limited antimalarial activity when tested against blood-induced McCoy strain vivax malaria. These data are summarized in Table VI. A daily dose of 3 grams for four days and a mean plasma drug level of 3.4 mgm. per liter were required to effect complete eradication of the trophozoites. A daily dose of 0.5 gram and mean plasma drug levels of 0.7 and 1.1 mgm. per liter had no effect. Intermediate doses and plasma drug levels had only partial effects. In contrast, daily doses of 1 gram of cinchonine for four days and mean plasma cinchonine levels as low as 0.1 mgm. per liter con-

sistently eradicated the trophozoites of the same strain of malaria (4). Daily doses of cinchonine as low as 0.5 gram and plasma drug levels less than 0.05 mgm. per liter exerted definite effects.

DISCUSSION

Equivalent plasma 2-hydroxy cinchonine levels are achieved on any given oral dosage of either cinchonine or of the 2-hydroxy compound itself. The plasma 2-hydroxy cinchonine levels are considerably higher than the corresponding plasma cinchonine levels. The differences in plasma cinchonine and 2-hydroxy cinchonine levels are not due to variations in gastro-intestinal absorption. Tissue localization was not specifically examined but it has been demonstrated in dogs that this factor is not important in determining variations in plasma drug concentrations among the cinchona alkaloids (11).

The binding on plasma protein is slightly less for 2-hydroxy cinchonine than for cinchonine, while the rate of renal excretion of the 2-hydroxy

TABLE V

Renal excretion of 2-hydroxy cinchonine on serial oral dosage of 2-hydroxy cinchonine

(Excretion values represent average of last three of four days on drug)

Daily dose	Number of subjects	Average per cent daily dose recovered
grams		
0.5	2	34
3.0	1	35
	Average	34

TABLE VI

Antimalarial activity of 2-hydroxy cinchonine
(Blood-induced McCoy vivax malaria—drug administered four days)

Subject	Daily dose	Mean plasma drug level	Class of therapeutic effect		
			I	II	III
	grams	mgm./L			
1	3.0	3.4			x
2	2.0	2.0			
3	2.0	1.5		x	
4	0.5	1.1	x		
5	0.5	0.7	x		

cinchonine is considerably higher than that of cinchonine. Both these factors should result in relatively lower plasma 2-hydroxy-cinchonine levels.

The differences in plasma drug concentrations, therefore, are probably the result of differences in the rates of metabolism. Cinchonine is apparently metabolized rapidly to the 2-hydroxy compound which in turn is metabolized at a slower rate. These rates of metabolism are reflected by the differences in the daily recoveries of cinchonine and its 2-hydroxy derivative from the urine of subjects given serial oral doses of cinchonine. That 2-hydroxy cinchonine is further metabolized is indicated by the recovery from the urine of only one-third of the administered doses and the recovery of sizable amounts of a further oxidation product (3). There appears, then, to be little doubt that 2-hydroxy cinchonine represents the first metabolic product of cinchonine and, further, that the main route of cinchonine metabolism goes through 2-hydroxy cinchonine.

Since the plasma 2-hydroxy cinchonine levels achieved in subjects given cinchonine were so much higher than those of cinchonine itself, it was of interest to determine the relative antimalarial activity of the 2-hydroxy derivative. On the basis of oral dosage, the 2-hydroxy cinchonine was only one-fifth as active as its parent compound. Furthermore, it required a mean plasma 2-hydroxy cinchonine concentration of 3.4 mgm. per liter to achieve the same antimalarial effect observed at mean plasma cinchonine levels of only 0.1 mgm. per liter.[†]

It may be concluded that the 2-hydroxy compound plays a negligible role in the antimalarial effect achieved when cinchonine is administered, in spite of the fact that much more of the metabolic product than of the parent compound is present at any given time. These observations, together with the demonstration that the main route of cinchonine metabolism proceeds through the 2-hydroxy derivative, indicate that cinchonine itself is the active antimalarial agent. Its rapid rate of metabolism, therefore, may be one of the important factors that limits its activity. This

situation does not necessarily hold for the other cinchona alkaloids.

SUMMARY

1. Of orally administered cinchonine, less than 5 per cent can be recovered in the urine as such, and more than 50 per cent, as the 2-hydroxy derivative.
2. Higher plasma 2-hydroxy cinchonine than cinchonine levels are achieved when cinchonine alone is administered or when equal doses of the two drugs are given.
3. The differences in plasma drug levels and urine excretions of cinchonine and 2-hydroxy cinchonine are chiefly the result of the more rapid rate of metabolism of cinchonine.
4. The main route of cinchonine metabolism proceeds through the 2-hydroxy derivative, its first metabolic product.
5. On the basis of plasma drug levels, 2-hydroxy cinchonine is only one-tenth as active an antimalarial as cinchonine.
6. The major portion of the antimalarial effect achieved when cinchonine is administered resides in cinchonine itself, and not in any of its metabolic products.

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[†] It is of interest to note that 2-hydroxy cinchonine is only one-fifth as active as cinchonine against *P. falciparum* trophozoites when assayed for antimalarial effect by a test tube technique (12).

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

V. THE ANTIMALARIAL ACTIVITY OF QUINACRINE^{1, 2, 3}

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INTRODUCTION

Although quinacrine has been used widely in the suppression and treatment of malaria since 1931, its usage up to and during the early years of the war was largely empirical insofar as dosage regimens are concerned. The development, in 1942, of simple reliable methods (1, 2) for the estimation of quinacrine concentrations in biological materials finally made possible studies of its physiological disposition in terms of measured drug concentrations (3). Information derived from these studies was essential in establishing a basis for the rational usage of quinacrine. Quantitative information concerning its antimalarial activity was equally important in the design of dosage regimens which permit the most effective use of this drug.

The observations reported in this communication are concerned with the suppressive antimalarial activity of quinacrine as measured by its ability to terminate clinical attacks of blood-in-

duced vivax and falciparum malaria. The results obtained describe the relationship between oral dosage, plasma quinacrine concentrations, and therapeutic effects. The range of effective plasma drug concentrations in infections due to selected strains of *P. vivax* and *P. falciparum* may be defined. In addition, the data provide a standard of reference for the comparison with quinacrine of promising antimalarial agents whose physiological disposition is similar to that of quinacrine.

PROCEDURE

The therapeutic tests were performed in accordance with standard procedures previously outlined (4, 5). The infections utilized were due to the McCoy and Chesson strains of *P. vivax* and the McClendon and Costa strains of *P. falciparum*. Quinacrine was administered by dosage schedules which produce fairly stable plasma drug concentrations during the four-day (vivax) or six-day (falciparum) therapeutic period. The therapeutic results are classified in three groups: Class I, no certain effect; Class II, a temporary suppression of parasitemia and/or fever; and Class III, a "permanent" effect, i.e., an absence of parasitemia for 14 days (vivax) or 21 days (falciparum) followed by a positive reinoculation to demonstrate continuing host-susceptibility to the infection.

In dealing with a drug such as quinacrine, which is localized extensively in tissues, whose rate of metabolic conversion is relatively low and which, consequently, persists in the plasma following the termination of therapy, it is difficult to obtain stable plasma drug levels which are limited to exactly four or six days. This difficulty was taken into consideration in the design of the therapeutic tests and was minimized by the adoption of two conventions. First, equilibrium plasma drug levels were achieved within the first few hours of therapy by the administration of suitable priming doses. Second, the actual period of drug administration was curtailed sufficiently to minimize the persistence of significant plasma drug concentrations beyond the desired four or six days. The mean plasma quinacrine levels presented in the tables are calculated from the levels obtained only during the first four

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A part of the material in this paper has been presented in a Harvey Lecture delivered by Dr. James A. Shannon on Oct. 25, 1945, and also appeared in "Survey of Antimalarial Drugs, 1941-1945," p. 177, J. W. Edwards, Ann Arbor, Mich., 1946. Permission to use Table I has been obtained from the Harvey Society and the editors of the Survey.

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or six days after starting therapy.⁶ The total oral doses are reported in terms of the free base and include priming as well as maintenance doses.

With potential Class III results, the selection of the time for reinoculation must be based in part on the number of days the drug persists in the plasma. It should be noted that in the standard procedure, the date of reinoculation is fixed with respect to "the day following the last effective plasma drug concentration" (4). Any plasma drug concentration which, when maintained for the period of the therapeutic test, produces a Class II result, is considered "effective." Consequently, follow-up observation periods began on the day when the plasma drug level fell below this value. In certain series of observations, Class II results were obtained at the lowest plasma quinacrine concentrations and the effective level,

TABLE I

The relationship between dosage and plasma concentration of quinacrine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

Patient	Total dose	Mean plasma quinacrine concentration	Class of therapeutic effect		
			I	II	III
	grams	µg./L			
Tra	0.59	44			x
Wal	0.59	39			x
Bel	0.59	34			x
Cra	0.59	32			x
Chu	0.59	31			x
Dar	0.72	27			x
Qui	0.59	27		x	
Wei	0.63	26			x
Wor	0.59	26			x
Cas	0.47	25			x
Daw	0.38	24		x	
Far	0.38	23		x	
Hau	0.51	22		x	
Mis	0.51	21			x
She	0.38	21		x	
Eic	0.38	19		x	
Mar	0.30	19		x	
Chi	0.38	18		x	
Kas	0.51	18		x	
Sch	0.59	18		x	
Ami	0.38	16		x	
Ash	0.63	16		x	
Ger	0.47	16		x	
Coo	0.30	15		x	
Vit	0.30	13		x	
DeM	0.30	13		x	
Sca	0.38	12		x	
Cou	0.30	10		x	
McB	0.38	8	x		
Saw	0.21	7	x		
Gra	0.21	6	x		
Kel	0.21	3	x		

therefore, could not be ascertained. In these cases, the observation period was begun when the plasma drug level had fallen well below that which was estimated to be effective. A delay in the time of reinoculation will not modify the ultimate experimental result since it has been shown that the development of immunity during a period of clinical latency is not appreciable (4).

RESULTS

Blood-induced vivax malaria

The effectiveness of various mean plasma quinacrine concentrations, during a four-day therapeutic period, was examined in 32 cases of blood-induced vivax (McCoy) malaria (Table I). The data reveal that Class III results, a "permanent" interruption of the infection, were observed in 10 subjects at plasma quinacrine concentrations ranging from 44 to 21 µg. per liter. Class II results, a temporary suppression of parasitemia and/or fever, occurred in 19 cases at concentrations ranging from 27 to 10 µg. per liter. Four subjects demonstrated no certain effect from quinacrine administration at concentrations between 8 and 3 µg. per liter. Therefore, the minimal "effective" plasma quinacrine concentration appears to lie between 8 and 10 µg. per liter. The division between Class III and Class II results is somewhat less sharply defined. However, if a plasma quinacrine concentration of 25 µg. per liter is accepted as representing the critical level, then only two of the 29 individuals constituting the two groups may be regarded as exceptions and these fall within a very limited range of plasma quinacrine concentrations (21 to 27 µg. per liter).

When the various groups of therapeutic effects are considered in terms of the total dose of quinacrine administered, Class III results were obtained with a total dose as low as 0.51 gram, Class II results, with a dose between 0.59 and 0.30 grams, and Class I results, with a total dose as high as 0.38 gram (Figure 1).

Very limited studies were obtained with a second strain of *P. vivax* (Chesson). Two patients with plasma quinacrine concentrations of 26 and 29 µg. per liter, respectively, maintained for four days, demonstrated only temporary effects. When therapy was extended to six days, two patients with plasma quinacrine levels of 28 and 36 µg. per liter showed Class III results, while a third,

⁶ Expression of plasma drug concentrations in other terms, such as the area under the entire plasma drug level curve, or this value divided by the number of days a significant drug level persisted, did not yield a better correlation between plasma level and effect, or between dosage and plasma level.

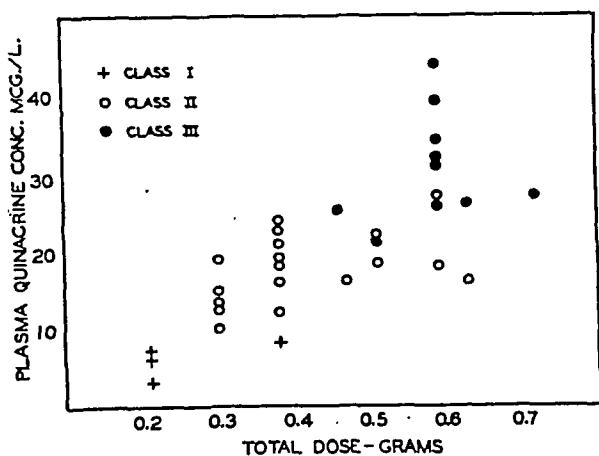


FIG. 1. RELATION BETWEEN TOTAL ORAL DOSE AND PLASMA QUINACRINE LEVEL AND ANTIMALARIAL EFFECT IN *P. VIVAX* (McCOY STRAIN) INFECTIONS

at 14 $\mu\text{g.}$ per liter, had only a Class II result. These results are in keeping with those obtained during quinine studies in the two strains (4) and indicate a greater resistance of the Chesson strain to quinacrine.

Blood-induced falciparum malaria

Observations on the effectiveness of quinacrine in blood-induced falciparum (McClendon) malaria were obtained in 24 subjects, stable plasma quinacrine concentrations being maintained for six days. It is apparent from the data presented in Table II that, in relation to plasma quinacrine concentrations, the division between Class III and Class II results was not as sharply defined as in vivax malaria. Plasma quinacrine concentrations ranging from 60 to 48 $\mu\text{g.}$ per liter resulted in four Class III effects. In the range between 47 and 22 $\mu\text{g.}$ per liter, there were six Class III and nine Class II results. All five subjects with concentrations below 22 $\mu\text{g.}$ per liter yielded Class II results. No Class I results were obtained in this series and, consequently, the minimal "effective" concentration was not ascertained.

There is even less correlation between the total oral dose of quinacrine and the therapeutic effect. One Class III result was achieved with a total dose as low as 0.59 gram, while Class II results occurred with total doses ranging from 0.59 to 0.93 gram.

A similar series of observations was obtained in 22 patients with blood-induced falciparum malaria due to a second strain (Costa). The results

TABLE II

The relationship between dosage and plasma concentration of quinacrine and therapeutic effect in six-day tests against blood-induced McClendon falciparum malaria

Patient	Total dose	Mean plasma quinacrine concentration	Class of therapeutic effect		
			I	II	III
	grams	$\mu\text{g./L.}$			
Ric	0.85	60			x
Rob	0.93	52			x
D'I	0.93	50			x
Smi	0.93	48			x
Hug	0.93	47			x
Alu	0.93	47		x	
Mon	0.93	45			x
DJO	0.85	38			x
Yea	0.85	32		x	
Mun	0.93	32		x	
Nev	0.93	29		x	
Tek	0.93	28		x	
Mev	0.85	27			x
Cru	0.59	27		x	
Ker	0.59	26		x	
Par	0.85	23			x
For	0.76	22		x	
Lad	0.59	22			x
Sta	0.80	22		x	
Wol	0.76	21		x	
Cia	0.93	19		x	
Cra	0.76	19		x	
Wil	0.59	14		x	
McK	0.76	9		x	

TABLE III

The relationship between dosage and plasma concentration of quinacrine and therapeutic effect in four-day tests against blood-induced Costa falciparum malaria

Patient	Total dose	Mean plasma quinacrine concentration	Class of therapeutic effect	
			II	III
	grams	$\mu\text{g./L.}$		
Fan	0.93	85		x
Wad	1.10	82		x
Che	0.89	76		x
Bry	1.00	74		x
Ber	0.89	68		x
Jor	0.93	66		x
Bro	0.89	65		x
Yee	0.89	61	x	
Fre	0.93	52	x	
Bax	0.93	51		x
Lem	1.01	50		x
Ron	0.93	49	x	
Sul	0.93	45	x	
Aus	0.93	34		x
Gwy	0.93	33	x	
Oli	0.89	32		x
Sim	0.93	28	x	
Par	1.10	27	x	
Mur	0.45	26	x	
AAR	0.89	20	x	
Bar	0.45	13	x	
Kou	0.45	13	x	

are summarized in Table III. All seven subjects with mean plasma quinacrine concentrations of 65 $\mu\text{g.}$ per liter or higher exhibited Class III results. In the range of 61 to 32 $\mu\text{g.}$ per liter, there were four Class III and five Class II results. All six subjects with plasma quinacrine concentrations of 28 $\mu\text{g.}$ per liter or lower exhibited Class II results.

From these data it seems likely that the Costa strain of *P. falciparum* is somewhat more resistant to the action of quinacrine than is the McClendon. This is supported by the observation that the lowest total dose of quinacrine which produced a Class III result in Costa falciparum was 0.89 gram as compared with 0.59 gram for the McClendon.

DISCUSSION

The therapeutic regimens of quinacrine in common usage prior to the war appear to have been constructed so as to produce a therapeutic effect roughly equivalent to that of quinine and at the same time minimize the incidence of toxic reactions. Although the usefulness of quinacrine attained wide acceptance, there were many clinicians who regarded quinacrine as inferior to quinine in the promptness with which an attack of malaria could be terminated. Pharmacological studies have since demonstrated that the extensive tissue localization of quinacrine delays the establishment of equilibrium between plasma quinacrine concentrations and oral dosage. Therefore, plasma quinacrine concentrations tend to be low during the first few days of therapy, unless suitable priming doses of quinacrine are administered (3). It was in consequence of this finding that a revision of therapeutic quinacrine regimens was recommended in 1943.⁷

The data presented show a positive correlation between plasma quinacrine concentrations and the suppressive antimalarial effects in both vivax and falciparum infections. The plasma quinacrine concentrations and the duration of therapy which are

required to interrupt permanently the erythrocytic phase of these infections vary from species to species and strain to strain. However, the data present a quantitative description of these factors in four infections with quite different biological and clinical characteristics. In the light of such information, it should be possible to appraise the adequacy of various quinacrine regimens recommended for the suppression and treatment of malaria.

In McCoy vivax malaria, a mean plasma quinacrine concentration of approximately 25 $\mu\text{g.}$ per liter, maintained for four days, will terminate an acute clinical attack of malaria, or, in other words, permanently interrupt the erythrocytic phase of the infection. In order to achieve a comparable result in Chesson vivax, a similar concentration must be maintained for six days. Both strains of *P. falciparum* examined appear to be approximately twice as resistant to quinacrine action as the Chesson strain of *P. vivax*, in terms of the plasma quinacrine concentrations required for a given clinical effect.

It has not been possible in such limited studies to determine the lowest plasma quinacrine concentration which exerts a demonstrable suppressive effect in each strain of plasmodium. The minimal effective concentration in McCoy vivax is 10 $\mu\text{g.}$ per liter when such a concentration is maintained for four days. Concentrations as low as 9 and 13 $\mu\text{g.}$ per liter have been shown to exert a definite suppressive action in McClendon and Costa falciparum, respectively, during a six-day period.

On the basis of these findings, it would appear that currently recommended dosage schedules yield plasma quinacrine concentrations adequate for the routine suppression and treatment of malaria. Subsequent experience has shown this to be so (6). However, it should be noted that the disposition of quinacrine in occasional individuals is such that unusually low plasma quinacrine concentrations are obtained. In these individuals, one may expect that the administration of quinacrine will result in suboptimal clinical responses.

The effectiveness of quinine in vivax and falciparum malarias has been examined in similar studies. Therefore, comparison of quinacrine and quinine in terms of effective plasma drug concentrations and the oral doses required to produce

⁷ According to Circular Letter No. 135, Office of the Surgeon General, Army M. Bull. (No. 65), pp. 216-218, January 1943, the recommended quinacrine dosage for continuous suppression is 0.1 gram once daily on six days of each week. For the treatment of clinical attacks, the recommended dosage is 0.2 gram every six hours for five doses, followed by 0.1 gram three times a day for six days (total 2.8 grams in seven days).

a given clinical response is possible. The plasma quinine concentration which will consistently produce a permanent interruption of the erythrocytic phase of McCoy vivax is approximately 5 mg. per liter (4) as compared with 25 μ g. per liter for quinacrine. A similar proportionality exists between the minimal concentrations of the two drugs which yield a barely detectable suppressive action. Thus, strictly in terms of effective plasma drug concentrations, quinacrine appears to be approximately 200 times as active as quinine in McCoy vivax infections. On the other hand, the oral doses of quinacrine which produce these critical plasma concentrations are only one-third to one-fourth of those required of quinine. A similar situation obtains in infections due to the McClendon strain of *P. falciparum*.

The validity of comparing the antimalarial activities of two agents on the basis of effective plasma concentrations alone may be seriously criticized. Perhaps a better basis of comparison of drugs falling into two quite different categories of physiological disposition is one in which a combination of the two factors is possible. The activity of a drug can be stated in terms of its "effective" concentration and its effective dosage by determining the mean daily dosage which is required to maintain the critical concentration. Taking the quinine level as 5 mg. per liter and the quinacrine level as 25 μ g. per liter, the relative activity of the two is approximately 500 mg./100 mg.

The general usefulness of plasma drug concentrations in the appraisal of antimalarial activity is emphasized by the data presented. In studies involving a single drug, the plasma concentration of the drug may be considered to be in equilibrium with the concentration at the site of action. In this sense, it is a more reliable datum than the oral dosage, especially with those drugs which are extensively localized in tissues. Furthermore, in the absence of data of this type, it would be diffi-

cult to construct rational dosage regimens for the routine management of malaria.

SUMMARY

The suppressive antimalarial activity of quinacrine has been examined in both vivax and falciparum infections induced by blood inoculation. The data presented describe the relationship between plasma quinacrine concentrations, oral dosage, and the therapeutic results, and define the range of effective plasma quinacrine concentrations in malarial infections due to selected strains of plasmodia. The susceptibility of the erythrocytic phase of malaria to quinacrine action varies with the species and strain of the offending organism. However, the information derived from these studies permits an appraisal of the adequacy of currently recommended dosage schedules for the suppression and treatment of malaria.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS. VI. THE PHYSIOLOGICAL DISPOSITION, ANTIMALARIAL ACTIVITY, AND TOXICITY OF SEVERAL DERIVA- TIVES OF 4-AMINOQUINOLINE ^{1, 2}

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One of the major contributions of the wartime malaria research program was the development of a number of synthetic antimalarial drugs with suppressive activity superior to that of quinacrine. Of the various chemical compounds examined, certain derivatives of 4-aminoquinoline are among the more promising.

The 4-aminoquinolines had been considered as potentially useful antimalarial agents prior to the war and, in fact, a number appear in the patent literature. Translated abstracts of the Russian literature deal with several members of this series (1). However, there is little evidence that these compounds had received adequate pharmacological, toxicological, or clinical study. One 4-aminoquinoline, SN-6911 (santochin), had been studied early in the course of the systematic survey of antimalarials in this country and had shown high activity in gallinaceum malaria in the chick (2, 3). However, the lead suggested by this observation was not appreciated at the time.

It was not until the French found SN-6911 to be well tolerated and to have high activity in the human malaras that a serious effort was made, in this country, to explore the 4-aminoquinoline se-

ries. A short time before, Blanchard had expressed interest in the potentialities of these compounds (4). A consideration of the chemical structure of quinacrine and related compounds led him to believe that derivatives of less complex nuclei should possess high antimalarial activity. He viewed the relationship of two such chemical series to quinacrine as shown in Figure 1.

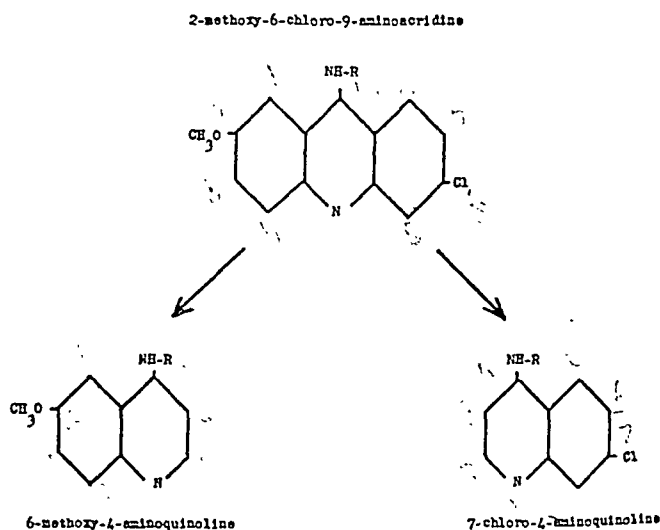


FIG. 1. RELATIONSHIP TO QUINACRINE OF TWO
4-AMINOQUINOLINE SERIES

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

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A large number of derivatives of 4-aminoquinoline have since been synthesized. These were examined for antimalarial activity in avian malaras and the most active were subjected to mammalian toxicity studies. The ten compounds shown in Table I have received trial in the human. It is the purpose of the present communication to report on studies of the physiological disposition, antimalarial activity and toxicity of several of these compounds.

TABLE I

Structure of the substituted 4-aminoquinolines studied
The antimalarial activity of SN-7373 and SN-10,960 was studied by the Boston group (5)

Survey number	Nuclear substituents	Substituent on 4-amino group
SN-3294	6-methoxy	diethylamino-1-methyl butyl
SN-6911	3-methyl-7-chloro	diethylamino-1-methyl butyl
SN-7135	2-methyl-7-chloro	diethylamino-1-methyl butyl
SN-7618	7-chloro	diethylamino-1-methyl butyl
SN-8137	7-chloro	diethylamino-2-hydroxy propyl
SN-9584	7-chloro	diethylamino propyl
SN-10,751	7-chloro	α -diethylamino-o-cresol
SN-13,425	7-chloro	1'-ethyl-4'-piperidyl
SN-10,960	7-chloro	diethylamino-3-hydroxy butyl
SN-7373	7-bromo	diethylamino-1-methyl butyl

CHEMICAL

The procedure developed for the estimation of each of the 4-aminoquinolines in biological materials involved the use of one of three general reactions.

1. The conversion of a non-fluorescent 4-aminoquinoline to a highly fluorescent substance by irradiation with near ultraviolet light (6). This is accomplished by irradiation of the compounds in an aqueous solution at pH 9.5 in the presence of cysteine to prevent further oxidation of the fluorophore formed. Several of the 4-aminoquinolines appear to yield the same fluorophore, since all of the compounds estimated with this procedure have the same relationship between quinoline concentration and intensity of fluorescence. This method was used for the estimation of SN-7618, 8137, 10,751, 7135, 13,425.

2. The formation of an organic-soluble, water-insoluble complex of the 4-aminoquinoline with methyl orange (7). This procedure was used for the estimation of SN-6911 only. The conversion of SN-6911 to a fluorophore requires radiant energy of a lower wavelength than was readily available. However, the rather high plasma drug concentrations obtained permitted the use of the less sensitive methyl orange reaction.

3. The direct measurement of fluorescence in an organic solvent (8). This method was used for the estimation of SN-3294 since the compound itself is highly fluorescent.

The evidence available indicated that the methods used for estimating the concentrations of the 4-aminoquinolines in biological materials are reliable and, in general, highly specific. The SN-3294 and SN-10,751 methods are exceptions in that certain metabolic products of the drugs are included in the final determination.

Section I

PHYSIOLOGICAL DISPOSITION

The data presented in this section describe some of the important aspects of the physiological disposition of the 4-aminoquinolines. Each compound was studied to obtain information relative to absorption from the gastro-intestinal tract, renal excretion, distribution in various tissues, binding on the non-diffusible constituents of plasma,

TABLE II

Absorption and excretion of several 4-aminoquinolines

Drug	Number of patients	Daily dose	Mean plasma concentration	Excretion			
				Stool		Urine	
		grams	$\mu\text{g./L.}$	mg./24 hrs.	percentage of daily dose	mg./24 hrs.	percentage of daily dose
SN-3294	2	0.4	560	15	4	88	22
SN-6911	3	0.6	449	15	3	140	23
SN-7135	2	0.3	197	22	7	62	21
SN-7618	2	0.4	217	31	8	57	14
SN-8137	2	0.3	181	4	1	40	13

and the effect of these discrete processes in determining the relationship between oral dosage and plasma drug concentration.

Absorption and excretion were examined in balance studies in which the subjects received a soluble salt of a drug over a period of days until plasma drug concentrations had become stable. Urine and stool collections were made during the last 48-hour period of drug administration (Table II). The small amount of drug recovered from the stools in each instance indicates fairly complete absorption from the gastro-intestinal tract. At equilibrium the urinary excretion, under ordinary conditions, accounts for 10 to 25 per cent of the daily oral dose. However, the rate of renal excretion can be made to vary over a wide range by the concurrent administration of acid or alkali, being increased by acid and decreased by alkali. Detailed studies relating acid-base balance and the physiological disposition of the 4-aminoquinolines are reported in a separate communication (9).

Data on the distribution of two compounds (SN-3294 and SN-7618) in the body fluids and

TABLE III

Distribution of quinacrine, SN-3294 and SN-7618 in the rat

Drug	Quinacrine	SN-3294	SN-7618
Days of drug administration	10	10	10
Plasma drug concentration, $\mu\text{g./L.}$	134	170	157
Tissue/plasma concentration ratio			
Erythrocytes	2.9	2.9	1.9
Whole blood	7.0	4.1	3.7
Brain	27.6	5.9	31.0
Muscle	194	77	41
Heart	1420	93	150
Kidney	3000	100	670
Lung	4070	150	640
Liver	6350	150	420

tissues of young adult albino rats are given in Table III. Quinacrine has been used as a reference drug. Groups of five animals received daily doses of 25 mg. of drug per kilogram by intubation for a total of ten days. Plasma and tissue samples for the estimation of drug concentrations were obtained 24 hours after the last dose. The group mean concentrations of drug in plasma and various tissues are presented in Table III. These describe the tissue/plasma concentration relationships which obtain in the therapeutic range of plasma drug concentrations.

It is apparent that the concentration of 4-aminoquinolines in tissues greatly exceeds that in the plasma, although less markedly than is the case with quinacrine. The relative concentrations in plasma, erythrocytes and whole blood demonstrate a limited localization in erythrocytes and a greater localization in leucocytes. The value of whole blood drug levels in quantitative pharmacological studies is obviously limited because of the variability of leucocyte counts. Similar results were obtained in the dog. In man, the plasma drug levels following a single intravenous injection of drug indicate that the distribution is qualitatively similar although the metabolic fate may differ somewhat from host to host.

Certain theoretical considerations dealing with relative plasma and tissue drug concentrations require information concerning the extent to which drugs present in the plasma are bound to non-diffusible constituents. The plasma binding of several 4-aminoquinolines and of quinacrine was measured in citrated human plasma at a pH of 7.35 and at 38° C. by dialysis in cellophane bags. The measurements were made at plasma drug concentrations known to be therapeutically effective. The data are summarized in Table IV. It is evident that all of the 4-aminoquinolines are

TABLE IV
Plasma binding of 4-aminoquinolines

Drug	Percentage bound to protein	Plasma drug concentration μg./L
SN-6911	75	700
SN-7135	43	90
SN-7618	55	80
SN-8137	48	105
SN-9584	50	90
SN-10,751	90	125
SN-10,960	52	90
Quinacrine	89	100

TABLE V
Relationship between plasma drug concentration and various daily oral doses of a series of 4-aminoquinolines

Drug	Daily dosage (mg.)			
	50	100	200	300
SN-3294	Mean plasma drug concentration, μg./L			
6911			107	112
7135				179
7618	22	49	110	176
8137	16	37	73	98
9584	19	37	68	126
10,751*	3.6	9.3	19	34

* Figures for SN-10,751 include an unknown fraction of degradation product.

bound to a considerable extent to the nondiffusible constituents of plasma, although SN-10,751 alone is bound to as great a degree as quinacrine. The percentage of drug bound *in vitro* varies widely and systematically with variation in pH, but it is doubtful that changes of practical importance occur within the restricted physiological range of pH observed in the human.

The plasma concentration of a chemotherapeutic agent, reflecting the oral dosage and the discrete physiological processes of absorption, distribution, metabolic degradation and excretion, provides a convenient means for following its over-all disposition. In addition, information describing the relationship between oral dosage and plasma drug concentration is necessary in the design of rational regimens of therapy. Data of this type, on each of the 4-aminoquinolines studied, are summarized in Table V for dosage schedules varying from 0.05 to 0.3 gram daily. With daily doses of 0.3 gram, the plasma drug concentrations of six of the drugs are of the same order of magnitude, ranging from 98 μg. per liter for SN-8137 to 179 μg. per liter for SN-6911. However, the concentrations of SN-10,751 are much lower and, in fact, considerably lower than indicated in the table inasmuch as it has been demonstrated that the measurement includes a fluorescent metabolic product. The SN-3294 method also lacks specificity, but there are no data available to indicate what proportion of the measured material is the parent compound.

Since the excretion of drug on serial oral dosage seldom exceeds 25 per cent of the total daily dose, it is apparent that the remainder of the drug undergoes metabolic degradation. No data are avail-

TABLE VI

Rate of disappearance of 4-aminoquinolines from plasma during five-day period

Drug	Number of patients	Plasma concentrations		Percentage remaining at 5 days
		3 hours after last dose	5 days after last dose	
		$\mu\text{g./L}$	$\mu\text{g./L}$	
SN-3294	5	214	51	24
SN-6911	4	220	50	23
SN-7135	4	201	56	27
SN-7618	6	28	15	53
SN-8137	10	31	9	29
SN-9584	6	25	10	40

able which describe the rates of degradation of the individual drugs. However, the rates of metabolic degradation and excretion, together with the degree of prior tissue localization, determine the rate at which plasma drug concentrations fall following the termination of therapy. Table VI contains data on the disappearance of the various drugs from plasma during the first five days after a complete course of therapy. All of the drugs studied are present in appreciable concentrations during this period, but SN-7618 has the greatest persistence in the body.

Comment. The 4-aminoquinolines studied are absorbed rapidly and more or less completely from the gastro-intestinal tract. They are localized in various tissues of the body, the degree of localization being intermediate between that of quinacrine and quinine (10, 11). Their excretion is greater than that of quinacrine but seldom exceeds 25 per cent of the total daily dose under ordinary conditions. The mean plasma drug concentrations achieved with standard oral dosage regimens are in the same order of magnitude for the drugs studied. Finally, all persist in the plasma in appreciable quantities for a number of days following the termination of therapy. In suppressive regimens with doses at long intervals, greater persistence as with SN-7618 may offer an advantage.

Section II

SUPPRESSIVE ANTIMALARIAL ACTIVITY

The observations described in this section are concerned with the ability of the various 4-aminoquinolines to terminate clinical attacks of blood-induced vivax and falciparum malaria. Additional

studies examine the suppressive activities of three of these drugs in experiments utilizing mosquito-induced vivax infections.

The values for activity obtained in these studies are not considered to be directly applicable to all strains of plasmodia. However, the tests in blood-induced infections utilized strains of *P. vivax* (McCoy) and *P. falciparum* (McClendon) whose susceptibility to quinine and quinacrine is known (12, 13, 14). The testing procedures have been demonstrated to yield reproducible results which define the chemotherapeutic susceptibilities of various strains. Consequently, the activity of related compounds may be compared and related to that of either quinine or quinacrine. Antimalarial activity assayed in this manner is probably a true measure of the ability of an agent to suppress, and to cure, naturally-occurring falciparum malaria and to suppress naturally-occurring vivax malaria.

Procedure. All of the therapeutic tests with blood-induced malaria were performed in accordance with standard procedures previously outlined (12, 13). The regimens of dosage were designed to produce fairly stable plasma drug concentrations during the four-day (vivax) or six-day (falciparum) therapeutic period as in the quinacrine studies (14). Therapeutic results are classified in three groups as previously defined: Class I, no certain effect; Class II, temporary suppression of parasitemia and/or fever; Class III, "permanent" effect, i.e., absence of parasitemia for 14 days (vivax) or 21 days (falciparum) followed by a positive reinoculation to indicate continued host-susceptibility to the infection. The separation of Class II and Class III effects must take into consideration the tendency of these drugs to persist beyond the termination of therapy. The conventions adopted in the design of therapeutic tests in such a situation have been discussed elsewhere (14).

Seven derivatives of 4-aminoquinoline have been tested for their effects on blood-induced infections with the McCoy strain of *P. vivax*. The results are presented in Table VII.

1. SN-3294. The study of this drug was limited to observations in eight patients because of its low antimalarial activity. It is the least active of the drugs, as judged either by the oral dosage or by the plasma drug concentration required to produce a given therapeutic effect. The smallest total oral dose which resulted in a Class II effect was 2.1 grams, while doses as large as 1.2 grams failed to exert any certain effect in two of the patients. The lowest mean plasma drug concentration producing a Class III effect

TABLE VII

Relationship between dosage, plasma drug concentration, and therapeutic effect in blood-induced vivax malaria (McCoy strain)

Drug	Total dose	No. of patients on dose	Mean plasma drug concentration ($\mu\text{g./L}$) classified according to therapeutic result		
			I	II	III
SN-3294	grams				
	2.7	2			338,321
	2.1	1			257
	1.3	2		110,134	
	1.2	2	41,47		
	1.1	1		91	
SN-6911	1.4	3			164,191,282
	1.0	2			130,191
	0.8	10		63,65,79,81	81,89,109,142, 150,174
	0.7	1			99
	0.6	2		54,76	
	0.35	1		54	
SN-7135	1.1	3			122,181,309
	0.6	2		51,65	
	0.3	1	26		
SN-7618	0.6	1			69
	0.55	1			42
	0.35	2			**
	0.30	6			14,16,18,20, 22,42
	0.275	1			13
	0.225	7	<1	8,10,10	5,11,*
	0.210	2		4,*	
	0.20	2			12,12
	0.13	3	5	2,5	
SN-8137	0.35	2			10,17
	0.30	6		9.5,12	21,24,27,31
	0.225	5		7,11,11,13,17	
	0.15	1		12	
	0.13	1		10	
SN-9584	0.375	4			8.5,14,23,28
	0.30	5		12,15	8.5,8.5,10
	0.225	4		8,9	10,14.5
SN-13,425	0.30	4		7	11,12,18
	0.225	8		5,6,7,9,9,9	10,14
	0.15	2	5,6	*,7	

* Plasma drug concentrations not determined.

was 257 $\mu\text{g.}$ per liter. Class I results were obtained with mean plasma concentrations as high as 47 $\mu\text{g.}$ per liter.

2. SN-6911. The relationship between dosage, plasma drug concentration, and antimalarial effect of SN-6911 in 19 patients with blood-induced McCoy vivax malaria is summarized in the table. The activity of this drug is comparable to that of quinacrine on the basis of the dosage required to produce a given effect, *e.g.*, at least 0.7 gram for Class III. However, the minimal plasma SN-

6911 concentration which results in Class III effects is approximately three times that of quinacrine.

3. SN-7135. This drug received only a limited study in six patients. Further work with the compound did not seem warranted since animal studies demonstrated its toxicity to be considerably greater than that of SN-6911.

4. SN-7618 (*Chloroquine*). Therapeutic tests with this drug in 25 patients are summarized in Table VII. It has the greatest antimalarial activity of the series, both in terms of dosage and of effective plasma concentrations. Total doses as low as 0.2 gram produced Class III effects. The critical plasma drug concentration which divides Class II and Class III effects is approximately 10 $\mu\text{g.}$ per liter, as compared with 25 $\mu\text{g.}$ per liter for quinacrine. Comparison with quinacrine by either standard of reference shows SN-7618 to be two or three times more active.

5. SN-8137. This compound is somewhat less active than SN-7618. The data from therapeutic tests in 15 patients are presented in the table.

6. SN-9584. The results of 13 tests are presented in Table VII. These reveal it to be a highly active compound. Although the relationship between oral dosage or plasma drug level and therapeutic effect is somewhat erratic within the narrow range of plasma concentrations achieved, it may be concluded that the antimalarial activity of SN-9584 is intermediate to those of SN-7618 and SN-8137.

7. SN-13,425. This drug was studied in 16 patients (Table VII). Its activity in man is approximately the same as that of SN-7618.

Four of the more active derivatives of 4-aminoquinoline⁴ were selected for trial in blood-induced falciparum malaria of the McClendon strain. The results of tests with the individual drugs are shown in Table VIII. The results in falciparum malaria need not be discussed in detail. As a group, the 4-aminoquinolines show the same high antimalarial activity in falciparum malaria that was demonstrated in vivax malaria. The relative activities of different members of the series would

⁴ This group of drugs includes SN-10,751 which was studied in McCoy vivax malaria by Dr. Allan Butler of the Harvard Medical School and which was found to have approximately the same activity as SN-7618 (5).

TABLE VIII

Relationship between dosage, plasma drug concentration, and therapeutic effect in blood-induced falciparum malaria (McClendon strain)

Drug	Total dose	No. of patients on dose	Mean plasma drug concentration ($\mu\text{g./L}$) classified according to therapeutic result		
			I	II	III
SN-6911	grams				
	1.7	4		103	124,142,152
	1.4	15	50	65,65,94,94, 95,130,140, 145,197	112,161,164, 166,181
	1.3	1			121
	1.2	1			71
	1.1	1			109
	0.9	1			115
	0.7	2		107,110	
SN-7618	1.0	3			95,113,128
	0.65	3			20,33,54
	0.35	10		12,16,18,18	15,18,19,22, 22,27
	0.275	1			16
	0.225	3		10,18	11
SN-8137	0.65	3			25,39,46
	0.475	1			37
	0.45	1			21
	0.425	2			27,28
	0.375	4		13,13,16	19
	0.25	1			19
SN-10,751*	0.8	2			36,35
	0.75	1		23	
	0.65	5		23,23,30	26,41
	0.375	1			28

* Plasma concentrations given are those of total fluorescent material.

appear to be in the same order in the two infections.

At a later date, and after information on the toxicity of these compounds was at hand, further study of their potential use in the routine suppression of malaria was undertaken. These experiments were designed to test the relative effectiveness of three of the more promising members of the group under experimental conditions simulating the use of the drugs as suppressives in the field. They were examined for their suppressive activity in mosquito-induced malaria due to the Chesson strain of *P. vivax*. Chesson vivax malaria differs from McCoy in at least two important respects: (1) The erythrocytic parasites are considerably less susceptible to the action of quinine or quinacrine, and (2) the mosquito-induced infections are characterized by frequent

true relapses beginning as early as one week after the termination of a full course of quinine therapy.

Thirty volunteer subjects⁵ were distributed at random into three equal groups. The drugs were administered in single doses of 0.25 gram once weekly for a total of four doses. Malaria was induced by the bites of *A. quadrimaculatus* mosquitoes infected with the Chesson strain of *P. vivax*. Each subject was bitten on three alternate days beginning one week after the first dose of drug, i.e., on the first, third, and fifth days of the second week of drug administration. Subsequent to biting, the salivary glands of the mosquitoes were dissected out, examined for the presence of sporozoites, and the positive glands graded 1 to 4 plus. The inocula, expressed as the total infection densities of mosquitoes biting each subject, ranged from 24 plus to 56 plus with an average of 41 plus. Thick blood smears were examined daily until the appearance of parasites. Blood samples for the estimation of plasma drug concentrations were obtained before and four hours after each drug dose and on the day parasites first appeared in the blood.

The results of this study are summarized in Table IX. With the exception of one subject in the SN-6911 group, all of those exposed to the bites of mosquitoes eventually developed clinical malaria.

TABLE IX

Comparison of suppression by weekly doses of 0.25 gram of SN-6911, SN-7618 and SN-8137 in mosquito-induced vivax malaria (Chesson strain)

	SN-6911	SN-7618	SN-8137
Average inoculum	43+	41+	39+
Ratio of patent infections to number exposed	9/10	10/10	10/10
Appearance of parasites:			
days after first inoculation			
Range	24-33	43-54	26-33
Average	29	50	29
Days after last dose of drug	15	36	15

Thick blood smears obtained between the 12th and 15th days after the first inoculation revealed small numbers of circulating parasites in three individuals in the SN-6911 group and three in the SN-8137 group. Fever did not accompany this demonstrable parasitemia. Thick blood smears failed to reveal parasitemia in any subject receiving SN-7618. However, when a suit-

⁵ This study was conducted at the United States Disciplinary Barracks, Green Haven, New York, with the cooperation of Colonel George Schulz, Lieut. Colonel Nathan Freeman, and Lieut. Colonel Michael D. Buscemi. The subjects were young, healthy volunteers.

able technique for the concentration of parasitized erythrocytes was applied to blood samples obtained during this period, as was to be expected, circulating parasites could be demonstrated in all of the subjects in the three groups (15, 16) except for the one member of the SN-6911 group who never developed clinical malaria. It must be presumed that inoculation had been unsatisfactory in this case.

The shortest prepatent periods⁶ in the SN-6911 and SN-8137 groups were 24 and 26 days, respectively. With both of these drugs, parasitemia occurred consistently within 33 days, the average being 29 days. In the SN-7618 group, the shortest prepatent period was 43 days and the average, 50 days. In relation to the last dose of drug, the mean parasite-free intervals were 15 days for SN-6911 and SN-8137 and 36 days for SN-7618. The plasma drug concentrations of all three drugs at the end of the prepatent period were below the minimal effective suppressive level of the drug studied. It appears likely that a weekly dose of 0.25 gram of any one of the three drugs is close to the amount required to provide effective suppression against vivax malaria.

Section III

TOXICITY

The observations described in this section relate to the toxicity of some of the more promising of the 4-aminoquinolines. Five of the more active drugs were studied to determine the daily dose required to produce symptoms when each is administered in progressively increasing dosage.

Materials and methods

Sixty-four volunteers⁷ were distributed at random into four equal groups receiving, respectively, SN-7618, SN-8137, SN-9584, and SN-10,751. Thirty-two additional volunteers⁸ were divided into two equal groups receiving SN-7618 and SN-13,425 respectively.

⁶ Transient parasitemias of low density are disregarded in this instance and the term "prepatent period" is used to indicate the interval from the first inoculation to the appearance of parasites at the onset of clinical malaria.

⁷ This study was conducted at the New Jersey State Reformatory, Rahway, New Jersey, with the cooperation of the late Commissioner W. J. Ellis and of Lieutenant W. E. Kulp. The subjects were young, healthy volunteers.

Each individual received an initial priming dose of the drug under study, followed by uniform dosage for a period of one week. The daily dose was increased in step-wise fashion at intervals of one week, each increase being preceded by a booster dose. The amount of drug administered each day was divided into two equal parts, and was taken in colorless capsules at 8 a.m. and 8 p.m. under direct supervision. All doses were calculated as the amount of the base administered. The schedule of dosage is shown in Table X.

TABLE X

Dosage schedule for toxicity study

During the first five weeks the dosage schedules were the same for all five drugs.

Week	Priming dose mg.	Daily dose mg.
1	200	50
2	200	100
3	300	200
4	400	300
5	600	400
6	None	*

* SN-8137, 600 mg.
SN-9584, 400 mg.
SN-10,751, 400 mg.
SN-7618, placebos.
SN-13,425, placebos.

The desirability of reporting all symptoms was emphasized at the start of the study and an opportunity to report symptoms was offered at the time each dose was administered. However, in order to minimize the factor of suggestion, the subjects were not questioned as to the presence or absence of specific symptoms, except to follow up symptoms previously reported. Each individual was interviewed at the termination of the study and questioned in detail as to his general state of well-being. An attempt was made to elicit any symptoms which had been noted during the period of drug administration.

Adverse reactions

There were sporadic complaints of various symptoms apparently unrelated to the medication throughout the period of the study. These symptoms were usually vague and followed no particular pattern in relation to the drug being taken by the individual. These complaints usually disappeared with the continued administration of increasing doses of medication. The symptoms of intoxication appeared later and showed striking uniformity within any one group.

SN-7618. Twelve of the 32 men who received SN-7618 were asymptomatic throughout the study. Only one subject developed symptoms directly attributable to the drug on a dosage less than 400 mg. per day. This individual complained of spells of light-headedness, dizziness,

and weakness while receiving 300 mg. of SN-7618 daily. The relationship of symptoms to drug was demonstrated by the disappearance of symptoms when placebos were substituted, and their prompt recurrence when medication was resumed.

Eye symptoms, occurring in 18 of the 32 members of the group, were the most striking and frequent toxic effect noted. These complaints appeared soon after the dose increased to 400 mg. per day. The exact nature of the symptoms was difficult to ascertain. Blurring of vision on looking from near to distant objects, which has been noted in other studies (17), was described definitely by one subject in this group. Most of the others were able to state only that there was something wrong with their eyes, or that they felt heavy, or their vision was blurred.

Generalized itching occurred in one man receiving 400 mg. daily.

All symptoms disappeared within a few days when SN-7618 was replaced by placebo capsules.

SN-8137. There were no symptoms attributable to SN-8137 in 16 men receiving up to 600 mg. of drug daily. A rash which was noted in one volunteer a few days after starting drug, disappeared during continued medication and seemed typical of pityriasis rosea.

SN-9584. Only two men who received SN-9584 remained completely asymptomatic throughout the study. One other had only transient symptoms early in the course of drug administration.

Itching was the most striking complaint of the volunteers receiving SN-9584. This symptom appeared in nine of the 16 men early in the fifth week of drug administration (400 mg. daily). The pruritus involved chiefly the forearms and legs, although in a few it extended to the neck, shoulders, and back. It was most severe at night and was noted by several individuals only at that time. No skin lesion other than excoriation was detectable in any member of the group. The pruritus persisted for variable periods of time after drug was discontinued, being present in some individuals up to three weeks later.

Nausea and vomiting were reported during the last two weeks by four members of the group, three of whom also had pruritus.

Marked nervousness, anxiety, and tremor occurred in three individuals. Two were receiving 400 mg. daily, while the other was receiving a

daily dose of only 200 mg. at the time these symptoms developed. In each case, improvement was noted within four to five days despite continued medication.

SN-10,751. Four of the 16 who received SN-10,751 were completely free of symptoms throughout the period of drug administration. Three others had minor complaints of brief duration at some time during the first three weeks of the study, but were free of symptoms during the final three weeks.

The symptoms noted in this group were somewhat ill-defined and non-specific and hence difficult to evaluate in the individual case. However, they did follow a fairly definite pattern and similar symptoms were relatively infrequent in the other groups during the latter part of the study.

Towards the end of the fourth week (300 mg. daily) eight men began to complain of lassitude, lack of energy, and inability to get to sleep at night. Several complained of a feeling of uneasiness in the epigastrium although they did not have definite nausea. Two individuals noted that, although they felt hungry between meals, their appetites disappeared on sitting down to eat. One other subject developed nausea and vomiting while receiving 200 mg. daily. This man had a past history of abdominal cramps and vomiting but it is interesting and possibly significant that the concentration of drug in his plasma was consistently the highest of any in the group.

Symptoms were sufficiently severe so that two men found it necessary to stop working during the last two weeks of the study and a third man refused further medication at the end of the fifth week.

SN-13,425. Six of the 16 men who received SN-13,425 were free of symptoms during the period of drug administration. Itching was the most frequent complaint among those volunteers who exhibited an intolerance to the drug. A total of seven subjects complained of generalized pruritus, four beginning in the third week, one during the fourth week, and two during the fifth week. A skin rash consisting of small, discrete, erythematous papules distributed over the arms, legs, buttocks and scrotum accompanied the itching in four subjects and persisted throughout the remaining period of drug administration.

During the fourth week, two of the subjects

complained of nervousness and a third developed an acute anxiety state. The latter reaction was characterized by flushing of the face, extreme nervousness, insomnia and tremor, which continued for more than one week despite the immediate cessation of therapy.

No significant eye symptoms were noted by the individuals receiving this drug.

DISCUSSION AND SUMMARY

The group of drugs derived from 4-aminoquinoline includes a number of compounds showing high antimalarial activity with potential usefulness in the treatment of human malaria. Changes in the nuclear substituents and changes in the character of the side chain are each accompanied by significant alterations in the physiological disposition, the antimalarial activity, and the toxicity of the resulting compounds.

The 4-aminoquinolines showing the most marked antimalarial activity in both avian and human malaria are derived from 7-chloro-4-aminoquinoline. The best combination of high activity and low toxicity was found in either SN-7618 or SN-8137. Both have advantages over quinacrine in their lower toxicity and in the smaller dosage or plasma drug concentration required to produce a given effect. Although SN-8137 is considerably less toxic than SN-7618, it is also somewhat less active. Administration of SN-7618 for over a year has shown it to be a safe suppressive agent when the recommended dosage is used (18). Either drug is useful for routine suppression, but it would appear from the data derived from the field-type suppressive study that SN-7618 has a considerable margin of efficacy because of its greater activity and persistence.

Dosage regimens which should be suitable for the suppression of malaria and for the treatment of acute attacks were chosen on the basis of the data presented in this paper. Work based on these suggested regimens has already been published (19, 20) and confirms the conclusions drawn from the experimental data.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS. VII. THE ANTIMALARIAL ACTIVITY OF PAMAQUINE^{1, 2, 3}

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Pamaquine, synthesized in 1926, was introduced in the treatment of malaria as a schizonticide. It was soon found, however, that the great schizonticidal activity which it possessed in cathemerium malaria of canaries did not obtain in the human malaras. It was shown to be relatively ineffective in acute attacks of vivax malaria and to have only minimal activity against the asexual forms of *P. falciparum*, although it did eradicate the gametocytes in this infection. In addition, the general usefulness of pamaquine was limited by the frequent occurrence of toxic effects with doses in the therapeutic range. Nonetheless, evidence was advanced favoring both a prophylactic (1, 2, 3) and curative action (4) in vivax malaria. The toxicity of the drug and an incomplete understanding of the biology of vivax malaria led the commission on malaria of the Health Organization of the League of Nations (5) to state that its prophylactic action was not practical and to discount the work of Sinton on its curative action (4). The commission recommended that pama-

quine be used only as a gametocide in falciparum malaria.

One of the objectives of the wartime malaria program was the development of agents which would cure vivax malaria. The discovery of drugs with suppressive activity greatly in excess of that of quinacrine made it possible to study the hypothesis that increased suppressive action might be sufficient to prevent the relapses of vivax malaria. With chloroquine (SN-7618) and paludrine it was possible to achieve and maintain concentrations of drug in the plasma many times that required to interrupt the acute attack and eradicate the erythrocytic parasites. True relapses of sporozoite-induced vivax malaria, however, were not eliminated. It was evident that chemotherapy directed against the tissue form of the parasite responsible for relapses (6) would require a qualitatively different action.

Independently in this country and in Great Britain, attention was directed to the older experiments which suggested that pamaquine did, indeed, have an effect on the tissue forms of vivax malaria. The experiments of James in the prophylaxis of mosquito-transmitted malaria were confirmed by Watson *et al.* (7). In Great Britain the effect of pamaquine, administered with quinine, in reducing the incidence of relapses in naturally acquired vivax malaria was again demonstrated (8).

Studies were undertaken in this laboratory to examine the pharmacology and toxicity of pamaquine, to determine more precisely its suppressive activity in vivax and falciparum malaria, and to explore its potentialities in the cure of mosquito-transmitted vivax malaria. The purpose of the study was not only to define the usefulness of pamaquine itself in the treatment of malaria in the human, but also to obtain information which might

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A portion of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 165 and 207.

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be valuable in the exploration of related compounds.

SUPPRESSIVE ANTIMALARIAL ACTIVITY

Methods. The ability of pamaquine to interrupt acute attacks of vivax and falciparum malaria was studied, using standard techniques previously described (6, 9). Infections with the McCoy strain of *P. vivax* and the McClendon and Costa strains of *P. falciparum* were established by the intravenous injection of infected blood containing a standard number of parasites. Dosage schedules were designed to produce fairly uniform concentrations of pamaquine in the plasma during the four- and eight-day test periods utilized in the vivax infections and during a six-day period in falciparum infections. The results of therapy are classified in three groups: Class I, no definite effect on the course of the infection; Class II, a temporary suppression, either partial or complete, of parasitemia and/or fever; and Class III, a complete interruption of the schizogonous cycle manifested by the absence of parasitemia for 14 days (vivax) or 21 days (falciparum) followed by a positive reinoculation to demonstrate continuing host susceptibility to the infection.

Blood-induced vivax malaria. The effect of various doses of pamaquine administered for four days was observed in 14 patients with blood-induced vivax (McCoy) malaria (Table I). The concentrations of drug in the plasma were determined by the method of Brodie *et al.* (10). Daily doses of 30 mg.⁶ or more resulted in com-

TABLE I

Relationship between daily dosage and plasma level of pamaquine and therapeutic effect in blood-induced vivax malaria (McCoy strain)

Four-day test

Patient	Daily dose	Mean plasma concentration	Result class		
			I	II	III
	grams (base)	μg./L			
Si	.09	571		x	
Ko	.09	422		x	
Ki	.09	196		x	
Bi	.06	127		x	
To	.06	84		x	
Ca	.03	114		x	
Me	.03	45		x	
Ra	.03	16		x	
Pi	.02	17		x*	
Le	.02	25		x*	
Fl	.01	22		x*	
An	.01	313		x*	
Pl	.004	9		x*	
Ow	.004	2	x		

* Partial effect on parasites.

⁶ All dosages are recorded in terms of the free base.

TABLE II

Relationship between daily dosage and plasma level of pamaquine and therapeutic effect in blood-induced vivax malaria (McCoy strain)

Eight-day test

Patient	Daily dose	Mean plasma concentration	Result class		
			I	II	III
	grams (base)	μg./L			
Go	.09	600		x	
Hu	.09	147			x
Ru	.02	23		x	
No	.02	20		x	

plete temporary suppression of both parasites and fever in every instance. Daily doses of 4–20 mg. produced partial or temporary suppression of parasitemia, except in one individual whose infection was not definitely affected by a daily dose of 4 mg. In no instance was a Class III (permanent) effect observed. The effect obtained was not as clearly related to plasma drug concentration as to the daily dose of drug.

The period of therapy was extended to eight days in four additional patients (Table II). Complete and permanent (Class III) interruption of the infection was produced in one subject who received a daily dose of 90 mg.

Blood-induced falciparum malaria. Observations on the suppressive activity of pamaquine in blood-induced falciparum (McClendon) malaria were obtained in eight individuals (Table III). The effect of 30 mg. daily for the six-day test pe-

TABLE III

Relationship between daily dosage and plasma level of pamaquine and therapeutic effect in blood-induced falciparum malaria (McClendon strain)

Six-day test

Patient	Daily dose	Mean plasma concentration	Result class		
			I	II	III
	grams (base)	μg./L			
VI	.06	545*		x	
Ga	.06	188		x	
Ed	.06	128		x	
An	.06	95		x	
Th	.06	72		x	
Wr	.03	89		x	
Sa	.03	74		x	
Wi	.03	36*		x	

* Patient received drug for only five days.

TABLE IV

Relationship between daily dosage and plasma level of pamaquine and therapeutic effect in blood-induced falciparum malaria (Costa strain)

Six-day test

Patient	Daily dose	Mean plasma concentration	Result class		
			I	II	III
	grams (base)	µg./L			
Ad	.06	2000		x	
Ma	.06	174		x*	
Bu	.06	92		x*	
Co	.06	215	x		
Ko	.06	39	x		

* Partial effect on parasites.

riod did not differ significantly from that of 60 mg. daily. In each instance fever was temporarily interrupted and the number of parasites diminished. Parasites were temporarily absent from thick blood smears in only one case.

Similar observations were made in five patients with blood-induced falciparum malaria due to the Costa strain (Table IV). Among five patients who received 60 mg. daily for six days, two showed no effect, two partial effects, and one a temporarily complete suppression of the infection.

CURATIVE ACTIVITY IN *P. vivax* INFECTIONS

The curative activity of pamaquine in mosquito-transmitted infections with the Chesson strain of *P. vivax* was examined in two series of young white male volunteers with no past history of exposure to malaria.⁷ Infections were established by the bite of mosquitoes infected with the Chesson strain of *P. vivax*.⁸ Each mosquito bit only one individual. The salivary glands of mosquitoes known to have bitten were removed by dissection and examined for the presence of sporozoites. The density of the gland infection was estimated as one to four plus. The inoculum received by each subject was recorded as the summation of the infection-densities of the mosquitoes by which he had been bitten. Thick smears were examined daily for the presence of parasites from the time of the expected termination of the prepatent period until the completion of drug therapy and thereafter at in-

tervals not exceeding one week. Drug therapy was instituted in primary attacks on the fourth day after the onset of fever above 100.6° F. (rectal). In later attacks therapy was started as soon as the presence of a relapse was confirmed. Dosage regimens were designed to produce and maintain a fairly constant plasma drug concentration throughout the period of drug administration.

The reappearance of parasites at any time after the completion of drug therapy was considered to represent a relapse.

The patients in the exploratory studies conducted at Goldwater Memorial Hospital were divided into five small groups as follows:

Group I (Controls)—Quinine alone for 16 days (2 grams daily), five patients.

Group II—Quinine for 16 days (2 grams daily) and pamaquine for 14 days (30 mg. daily), the latter beginning on the third day of quinine therapy, four patients.

Group III—Quinacrine for six to 16 days (0.3 gram daily) and pamaquine for 14 days (30 mg. daily), the two drugs being started simultaneously, four patients.

Group IV—Quinine for 16 days (2 grams daily) and pamaquine for 14 days (90 mg. daily), the latter beginning on the third day of quinine therapy, nine patients.

Group V—Pamaquine for 14 days (90 mg. daily), followed by quinine for 10 days (2 grams daily), five patients.

A summary of the results of these experiments is given in Table V. It may be concluded from these data that a combination of pamaquine and quinine has a curative action in Chesson vivax malaria, providing the drugs are administered concurrently in high dosage and for a long period of time. The groups are too small and the variables too many, to warrant further analysis of the data.

Other studies were conducted by the University of Chicago group at Stateville (11) and by ourselves at the U. S. Disciplinary Barracks, Green Haven, in order to determine more accurately the quantitative nature of this curative action.

The patients in the Green Haven studies were divided into two groups. Twelve volunteers were treated in their primary attacks as well as in each subsequent relapse, with 2 grams of quinine daily for 16 days. The primary attacks in 18 subjects were treated with quinine, 2 grams daily for 16 days, plus pamaquine, 60 mg. daily for 14 days, beginning on the third day of quinine therapy. Each subsequent attack in these individuals was treated with the same regimen. This dosage of

⁷ A group of 27 conscientious objectors was assembled at Goldwater Memorial Hospital through the cooperation of the American Friends Service Committee and the Selective Service Administration. Arrangements for the study conducted at the United States Disciplinary Barracks, Green Haven, New York, were made through the cooperation of the Office of the Surgeon General.

⁸ Many of the infected mosquitoes were furnished by Doctors Clay G. Huff and Frederick Coulston, whose assistance is gratefully acknowledged.

TABLE V

Curative effect of pamaquine in primary attacks of sporozoite-induced vivax malaria (Chesson strain)

Patient	Inoculum*	Mean drug concentration		Days to relapse	Observation period (negative cases)
		Quinine	Pamaquine		
		mg./L	µg./L		days
Group I—Quinine alone					
Ho	8+	10.8	—	426	463
Bu	14+	10.3	—	12	
Ba	11+	8.9	—	8	
St	10+	8.4	—	21	
Ma	7+	8.1	—		
Group II—Quinine and pamaquine (30 mg. daily) concurrently					
		Quinine	Pamaquine		
Ni	3+	9.1	69	361	331
Ja	6+	10.3	61		
Do	6+	11.2	51		10
Ha	11+	7.3	41		330
Group III—Quinacrine and pamaquine (30 mg. daily) concurrently					
		Quinacrine	Pamaquine		
Ca	12+	66	311		334
Bra	5+	81	290		334
Bro	13+	61	261		358
Hu	8+	69	209		314
Group IV—Quinine and pamaquine (90 mg. daily) concurrently					
		Quinine	Pamaquine		
Sw	8+	11.2	264		552
Fl	17+	12.9	198		555
Le	17+	10.4	186		551
Bu	18+	10.6	178		552
Ro	11+	11.6	168		547
Wi	20+	10.6	167		508
Cl	11+	9.3	154		548
Mo	13+	11.1	132		548
Sc	16+	9.3	110		551
Group V—Pamaquine (90 mg. daily) followed by quinine					
		Quinine	Pamaquine		
Br	9+	5.4	265	51 55	457
Go	13+	9.5	112		459
No	10+	5.4	110		459
Ed	12+	5.4	76		
We	12+	6.7	71		

* Summation of individual mosquito infection densities.

pamaquine was selected as being the most likely to produce a distribution of cures and non-cures based on the information available from the Goldwater studies.

The results of treatment in these two groups are presented in Tables VI and VII. The short and uniform interval between treatment with quinine alone and relapse is strikingly shown in Table VI. The results in this group differ considerably from those obtained in the patients similarly treated at Goldwater Memorial Hospital (Group I, Table V). It is believed that the difference is attributable largely to the heavier sporozoite inoculum used in the Green Haven studies.

The results of treatment with simultaneous pamaquine (60 mg. daily) and quinine differ sharply from those obtained with quinine alone. Although 13 of the 18 subjects had at least one relapse, the interval from treatment to first relapse was markedly prolonged and variable. The incidence of second relapse was markedly reduced and only one-third relapse was observed. Within any group of similarly treated patients there was no relationship between plasma drug concentration and the occurrence of relapse.

DISCUSSION

The suppressive action of pamaquine is more marked in vivax than in falciparum malaria but there exists at least as much difference between different strains of falciparum as between vivax and falciparum. However, it is evident that, in doses which approach the maximum tolerated, pamaquine is a relatively ineffective suppressive drug against both vivax and falciparum malaria, being, in general, incapable of effecting a complete interruption of the schizogonous cycle. For this reason, in studies designed to test the curative action of pamaquine, the erythrocytic phase of vivax malaria must be eradicated with a more potent suppressive agent. This is necessary to insure that each recurrence of activity represents a true relapse rather than the appearance of a temporarily suppressed erythrocytic phase.

Pamaquine has a curative action in vivax malaria which is clearly apparent when the drug is given at high dosage, in combination with quinine and for a period of 14 days. Pamaquine administered alone has a curative action but this is not

TABLE VI

Interval from treatment with quinine to relapse in individuals with sporozoite-induced vivax malaria (Chesson strain)

Each attack was treated with 2 grams of quinine (base) daily for 16 days

Patient	Inoculum	Primary		1st relapse		2nd relapse		3rd relapse		4th relapse	
		Mean quinine level	Days to relapse	Mean quinine level	Days to relapse	Mean quinine level	Days to relapse	Mean quinine level	Days to relapse	Mean quinine level	Days to relapse
		mg./L		mg./L		mg./L		mg./L		mg./L	
Ci	29	8.5	8	7.3	10	6.8	17	*		*	
St	22	8.3	9	6.1	8	5.8	13	7.6	13	*	
Mo	47	6.8	9	6.1	8	*					
Pe	35	6.5	6	5.7	12	6.1	13	*			
Mo	28	6.3	9	4.8	13	4.2	17	5.7	22	*	
Ra	21	6.1	5	5.0	11	4.5	11	3.4	9	*	
He	47	6.0	9	4.9	8	6.1	9	*			
Pa	47	5.9	9	4.9	10	5.4	11	5.0	37	*	
Wi	41	4.9	6	3.5	7	5.4	7	*			
Ph	19	10.0	8	6.4	13	5.5	17	3.8	17	*	
Do	21	7.0	9	6.4	9	5.8	11	*			
Du	32	5.8	8	5.5	8	6.0	12	*			

* Study discontinued.

clearly defined by the experimental data presented here (cf. group V, Table V). The data which bear on the curative action of pamaquine also

TABLE VII

Interval from treatment with combined quinine and pamaquine to relapse in individuals with sporozoite-induced vivax malaria (Chesson strain)

Each attack was treated with 2 grams of quinine (base) daily for two days, followed by concurrent administration of 2 grams of quinine and 60 mg. of pamaquine daily for 14 days.

Patient	Inoculum	Primary attack			First relapse			Observation period (negative cases)
		Mean quinine level	Mean pamaquine level	Days to relapse	Mean quinine level	Mean pamaquine level	Days to relapse	
		mg./L	µg./L		mg./L	µg./L		days
Mc	35	7.6	216					256
Mo	35	6.2	208	62	5.4	188		214
Cr	26	8.9	128	69	7.5	105		201
Ro	31	7.0	116	24	6.8	120		251
Ada	32	7.8	109					276
No	25	6.7	108	67	6.2	82	80*	
Adl	21	5.8	80	26	4.1	48		249
Du	29	8.5	72	20	5.8	56		250
Au	22	6.7	70					277
Be	22	6.6	70	22	5.6	47		218
Go	54	6.5	67	50	4.8	53		199
St	30	6.3	63	22	5.3	41		253
Di	25	5.7	63	47	6.0	68		221
Si	19	6.5	62	22	5.8	62		248
Do	24	5.6	60					276
Tr	26	8.0	56					256
Gi	37	5.2	42	54	4.5	50		215
Na	32	6.9	113	21	6.3	74	51**	

* Study discontinued.

** This patient had a third relapse, 129 days later; the mean plasma quinine concentration was 4.7 mg./L; the mean plasma pamaquine concentration was 71 µg./L.

demonstrate that the tendency of vivax malaria to relapse and the time of appearance of the relapse, whether treated with quinine alone or with quinine and pamaquine, are in no small measure determined by the density of the initial infection (sporozoite dosage Tables V, VI, VII). Presumably, this is an important factor in determining the history of the disease when acquired naturally.

The data define the curative dosage of pamaquine for Chesson malaria in terms of the amount required to cure primary attacks resulting from heavy sporozoite dosage. This would appear to be in the range of 60-90 mg. of pamaquine base when administered in conjunction with 2.0 grams of quinine for 14 days.

It seems likely that curative effects observed at lower dosages (8, 12) are attributable to greater strain susceptibility, to the administration of drug at a later stage of the disease, or to a lesser density of the underlying tissue infection. The latter two circumstances may, in fact, be related. That is, a lesser density of the underlying tissue phase of the disease may result from either a smaller sporozoite inoculum or a diminution with time of an initially high density of tissue parasites.

Contrary to the situation encountered in the study of a number of suppressive antimalarials (13, 14, 15), there is no correlation between plasma pamaquine concentration and antimalarial effect whether suppressive or curative. Furthermore, a suppressive action is observed at very

low dosage but is not greatly increased with progressive increases in dosage to a very high level. Of interest in this relation is the observation that pamaquine has activity in developing cultures of erythrocytic parasites only at plasma drug concentrations many times those which are attained with doses in the usual range (16).

SUMMARY

1. Pamaquine at maximum tolerated doses is incapable of effecting interruption of the erythrocytic schizogonous cycle of vivax and falciparum malaria.

2. Pamaquine has a curative action in primary attacks of sporozoite-induced Chesson strain vivax malaria when the drug is given at high dosage, 60-90 mg. daily, in conjunction with 2.0 grams quinine daily for 14 days.

3. It appears that curative effects observed by other workers at lower dosages are attributable to greater strain susceptibilities, to administration of drug at a later stage of the disease, or to a lesser density of underlying tissue infection.

4. There is no correlation between plasma pamaquine concentration and antimalarial effect whether suppressive or curative.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS. VIII. THE PHYSIOLOGICAL DISPOSITION OF PAMAQUINE^{1, 2, 3}

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The recent appreciation (1, 2) of the earlier studies on the curative activity of pamaquine in mosquito-transmitted vivax malaria (3) has promoted renewed interest in the chemotherapeutic potentialities of this compound and of several related derivatives of 8-aminoquinoline. Consequently, a report on the physiological disposition of pamaquine seemed desirable.

METHODS AND MATERIAL

The individuals used in these studies were, for the most part, patients with central nervous system syphilis. Certain of the pharmacological studies were carried out in normal volunteers. Pamaquine disposition was usually studied prior to induction of therapeutic malaria or after convalescence had become well established. Some of the data on the relationship of dosage⁵ to plasma drug level, however, were obtained during the course of treatment for acute vivax or falciparum malaria. The chemical methods used have been described elsewhere (4).

RESULTS

I. Physiological disposition

A. Absorption. The absorption of pamaquine from the gastro-intestinal tract cannot be examined by balance experiments since the compound

is rapidly destroyed when incubated at 37° C. in a suspension of feces. However, indirect evidence of rapid and complete absorption is available from the comparison of curves of the concentration of pamaquine in plasma following single oral and intramuscular doses of the drug (Table I). The

TABLE I
Plasma pamaquine levels three hours after administration of 20 mg. (base) of the drug

Patient	Plasma pamaquine level after oral dose μg./L	Plasma pamaquine level after intramuscular dose μg./L
Sut	64	84
	69	84
	78	
	75	
Des	112	105
	112	77
Bow	94	91
	120	131
	121	
Hur	191	157
	198	170

peak concentration is higher and is reached earlier after intramuscular injection. Yet, at the end of three hours, the concentration is approximately the same, regardless of route of administration. Furthermore, differences in solubility between the various salts of pamaquine do not seem to condition the rate of absorption (Table II).

B. Distribution. The distribution of pamaquine in the body has been determined by direct analysis in the dog (Table III). There is some localization of the drug in the liver, lungs and brain, relatively less accumulating in other organs. Indirect evidence of a similar localization in the human was obtained. For example, immediately upon termination of a 15-minute infusion of 20 mg. of the drug, only 5 to 6 per cent can be accounted for in the circulating blood. Such a disappearance cannot be accounted for by metabolism and excretion and must represent localization.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A portion of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 185.

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⁴ Captain, MC, AUS.

⁵ All doses are given in terms of the free base.

TABLE II

Plasma pamaquine levels following oral administration of the hydroiodide and naphthoate salts of pamaquine

In each instance the dose furnished 20 mg. of the base.

Time (minutes)	Plasma pamaquine levels (micrograms per liter)			
	Patient Sin		Patient McK	
	Hydroiodide	Naphthoate	Hydroiodide	Naphthoate
30	0	4	8	3
60	8	14	24	31
120	97	69	42	66
180	90	69	69	69
300		28		41
480		14		17

C. Excretion. On serial oral dosage of 60 mg. daily, about 1 per cent of administered pamaquine was found in the urine of five patients.

D. Metabolism. The pathway of metabolism of the drug is not known. Evidence for the presence of two metabolic intermediates has been obtained from biological fluids of patients receiving pamaquine. These data will be presented in another communication (5). Pamaquine undergoes very rapid metabolic alteration. Eight hours after administration of 20 mg. of pamaquine, only negligible amounts can be detected in plasma, and this is not due to marked localization or excretion. However, as will be discussed below, the rate of disappearance from plasma may be greatly modified by quinacrine.

TABLE III

Distribution of pamaquine in the dog

Blood and tissues were obtained for analysis three hours after the dog received a single oral dose of 10 mg. per kilo of pamaquine (base) as the hydroiodide.

Tissue	Tissue pamaquine concentration ($\mu\text{g.}/\text{kilo}$)	Ratio tissue pamaquine concentration to plasma pamaquine concentration
Plasma	610	
Whole blood	814	1.3
Liver	22,900	37.6
Muscle	1,360	2.2
Brain	6,460	10.6
Spleen	725	1.2
Lung	15,830	26.0
Heart	3,690	6.0

II. Relationship between dosage and plasma concentration

A. Oral dosage. The plasma drug concentrations observed in a series of patients receiving either

30 or 60 mg. of pamaquine daily are presented in Table IV. The values in this table represent arithmetic means of plasma drug concentrations obtained just before and three hours after one of the daily doses. The detailed data bear out the suggestion carried in Table II with respect to the rapid rise and fall of plasma drug concentration. When pamaquine is administered at intervals as great as eight hours, peak concentrations are observed two to four hours after each dose, and the minimal concentrations at the end of eight hours are negligible at a daily dosage of 30 mg., and very small at 60 mg. Such rapid fluctuations in plasma drug concentration may be minimized by administration of divided doses at intervals no longer than four hours. There is a three- to four-fold variation in mean plasma concentration in a group of individuals on the same dosage schedule (Tables I, II and IV).

Occasionally, striking departures of plasma pamaquine concentration from the usual range have been observed. Patient Add, who received 10 mg. every four hours, had a mean plasma concentration of 2000 $\mu\text{g.}$ per liter for the six-day pe-

TABLE IV

Plasma pamaquine levels in a series of individuals receiving pamaquine orally daily in divided doses

The plasma pamaquine levels given below are the means of minimal and maximal plasma drug concentrations. The former is taken just before a dose, the latter three hours after a dose.

Patient	Dosage			Mean daily plasma pamaquine concentration on day					
	First day	Daily	Duration	1	2	3	4	5	6
	mg.	mg./hr.	days	$\mu\text{g.}/\text{L}$	$\mu\text{g.}/\text{L}$	$\mu\text{g.}/\text{L}$	$\mu\text{g.}/\text{L}$	$\mu\text{g.}/\text{L}$	$\mu\text{g.}/\text{L}$
Gom	60	15/4	8	233	925	1680	760	381	340
Gar	60	10/4	6	99	168	166	240	268	185
Edw	60	10/4	6	68	145	190	146	127	95
Ang	60	10/4	6	112	173	108	79	55	45
Add	60	10/4	6	1200	2057	2924	2435	1985	1400
Tho	60	10/4	6	60	81	54		76	89
Vlh	60	10/4	5	222	321	998	1031	543	163
Dad	40	5/4	5	128	100	100	83		
Wri	20	5/4	6	42	69	87	195	90	53
Sac	20	5/4	6	29	53	77	117	98	69
Won	40	5/4	4	87	74	60	55		
Ken	40	5/4	4	28	22	17	24		
Rob	20	10/8	6	25	105	84	63	76	63
Cha	20	10/8	6	42	59	46	44	33	14
Wil	20	10/8	5	34	23	44	40	72	
Chu	20	10/8	6	25	21	30	43	27	37
Dia	20	10/8	5	29	18	24	24	24	
Rod	20	10/8	6	21	29	25	17	17	25
Mes	20	10/8	6	13	21	25	23	21	25
Ant	10	2.5/6	4	270	402	358	213	27	

riod and, on the third day, had a level of 2924 μg . per liter. Patient Vlh, on the same dosage schedule, had a mean plasma concentration of 545 μg . per liter for the five-day period, and, on days 3 and 4, achieved levels of 1000 and 1030 μg . per liter respectively. Patient Gom, who received 15 mg. every four hours for eight days, reached plasma drug concentrations of 925, 1680 and 760 μg . per liter on days 2, 3, and 4 respectively, with levels in the expected range on the other days of the period. One patient, receiving only 2.5 mg. every six hours for four days, had a mean drug concentration for the four-day period of 313 μg . per liter and, on one day, had a level of 402 μg . per liter.

Instances such as these were exceptional and

were not due to prior or concurrent quinacrine therapy (*vide infra*). These patients were receiving no medication other than pamaquine.

B. Intramuscular dosage. Following the intramuscular administration of 20-mg. doses of pamaquine the maximum concentration in plasma was reached earlier than was the case after oral administration, usually in from 30 to 60 minutes, and was higher, ranging from 200 to 400 μg . per liter. At the end of three hours and thereafter, however, the plasma drug concentration was approximately the same as when the oral route was employed (Table I). Variability in plasma pamaquine concentration from patient to patient was noted after intramuscular as well as after oral administration. As in the case of orally adminis-

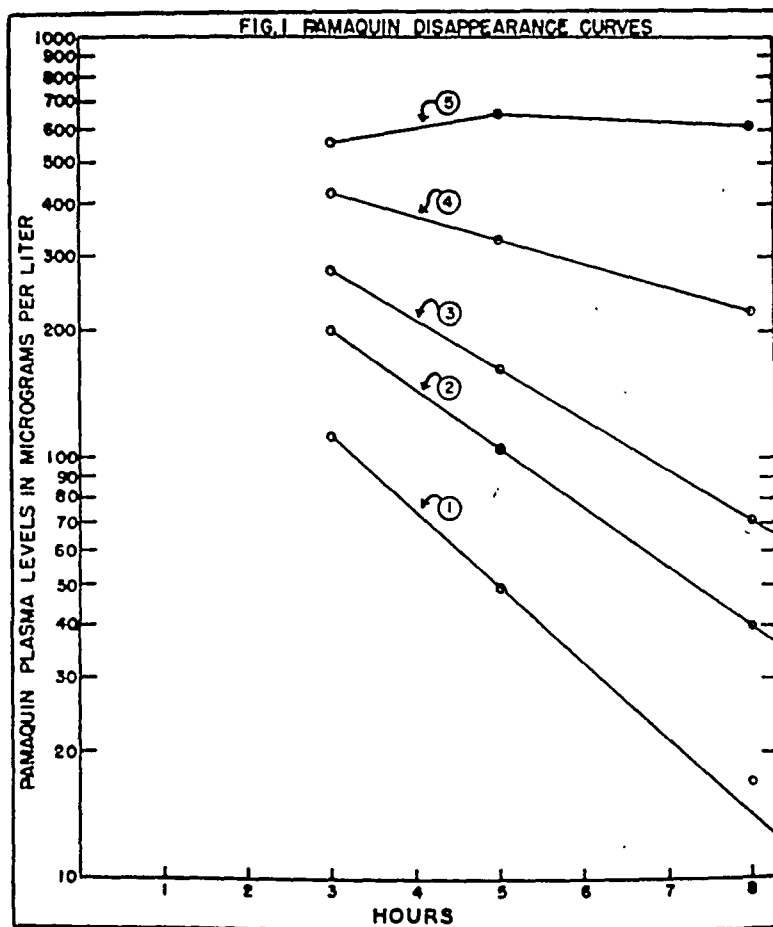


FIG. 1. EFFECT OF 10 MG. QUINACRINE EVERY EIGHT HOURS FOR EIGHT DOSES ON PLASMA PAMAQUINE CURVES FOLLOWING SINGLE ORAL DOSES OF 20 MG. PAMAQUINE (BASE)

Curve 1 = control; curve 2 = ten hours after first dose of quinacrine; curve 3 = one day after first dose; curve 4 = four days; curve 5 = eight days.

tered drug, plasma pamaquine concentration curves were reproducible in any individual when serial studies were made.

III. Modification of physiological disposition of pamaquine by other drugs

A. Quinacrine. Among individuals who achieved and maintained unusually high plasma pamaquine concentrations, the majority had previously received quinacrine. Systematic studies to determine whether quinacrine was causally related to the abnormal plasma pamaquine levels were carried out. This study was simplified by the fact that in any individual, the curve of plasma drug concentration was reproducible following standard 20-mg. doses of pamaquine.

Plasma pamaquine curves determined in the same individual before and after receiving various doses of quinacrine (Table V) showed that (1) the maximal plasma pamaquine concentrations are many times higher following quinacrine therapy than the control values and (2) the rate of disappearance is markedly reduced, as shown by the time necessary for the disappearance of 50 per cent of the maximum concentration (Table V).

TABLE V

Effect of quinacrine on plasma pamaquine levels after single oral doses of 20 mg. of pamaquine (base)

Patient	Quinacrine prior to observation	Maximum plasma pamaquine level		Time for 50% disappearance	
		Control	After quinacrine	Control	After quinacrine
	grams	$\mu\text{g./L}$	$\mu\text{g./L}$	hours	hours
To	2.5	112	660	1.7	23.0
Bo	2.0	62	575	2.0	7.7
Ke	0.5	63	425	1.8	8.1
Ai	1.4	86	605	3.0	5.2

In Figure 1, the control curve, number 1, and a series of curves performed in the order numbered at various intervals after initiation of quinacrine administration to patient To, are plotted. This figure shows both progressive upward displacement of the maximum and progressive retardation of the rate of disappearance after quinacrine was administered. The three other patients who were subjected to this type of study had curves in which similar changes were induced by quinacrine administration. Very small amounts of quinacrine are capable of producing this phenomenon. Curve number 2 of Figure 1 was performed ten hours after the first dose (20 mg.) of quinacrine, yet the

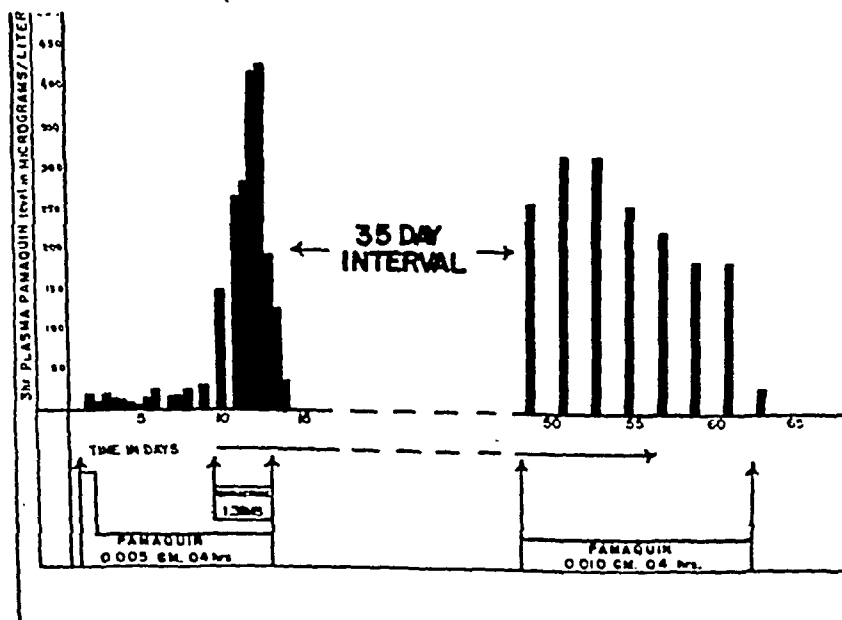


FIG. 2. EFFECT OF QUINACRINE ADMINISTRATION ON MEAN PLASMA PAMAQUINE LEVELS RESULTING FROM SERIAL ORAL DOSES OF PAMAQUINE

maximum has been doubled and the rate of disappearance retarded.

It is well known that quinacrine disappears slowly from the body. It would seem reasonable, therefore, to predict that the ability of quinacrine to modify pamaquine metabolism would not be an evanescent phenomenon. Daily pamaquine levels three hours after the 6 a.m. dose in an individual on constant pamaquine intake of 30 mg. daily are shown in Figure 2. During the control period, the daily plasma pamaquine levels averaged 40 μ g. per liter. During a period when 30 mg. of quinacrine was also administered daily, the plasma pamaquine levels were greatly elevated, reaching as high as 450 μ g. per liter. After a medication-free interval of 35 days, resumption of a slightly higher constant pamaquine dosage regimen resulted in plasma drug levels far in excess of the usual range and not explainable on the basis of increased dosage. Such effects have been observed as long as three months following the last dose of quinacrine in many patients.

Mean plasma pamaquine concentrations in four patients who received concurrently 30 mg. of pamaquine and 300 mg. of quinacrine daily were 100 per cent higher than the mean levels of patients who received 90 mg. of pamaquine and 2.0 gm. of quinine daily.

In none of these experiments were there any significant deviations of plasma quinacrine concentration from the expected levels.

B. Quinine. The effect of concurrent administration of quinine on plasma pamaquine concentration was investigated by experiments similar to those which demonstrate the effect of quinacrine. When concentration curves of pamaquine in plasma were studied in individuals before and during quinine administration, no significant alteration of either the height or the form of the curve was observed. These curves were analyzed after oral pamaquine administration in six cases, after intramuscular, in four cases, and after intravenous, in one case.

The introduction of quinine into the dosage regimen of individuals receiving 30 mg. of pamaquine daily was studied to determine whether the three-hour plasma pamaquine level was affected. In three cases there was a slight but probably significant rise in the plasma pamaquine concentration during the period of concurrent quinine

administration, while in one case, the plasma pamaquine level was not significantly changed. Analysis of the data collected from routinely determined plasma drug concentrations in individuals receiving quinine and pamaquine simultaneously for treatment of vivax malaria showed no constant relationship between the plasma concentration of one drug and that of the other.

The data collected in this laboratory do not permit a definitive answer to the question of the effect of quinine on pamaquine plasma concentrations. It can be said, however, that quinine does not have as marked an effect as does quinacrine, and that in all probability its effect, if it exists, will be demonstrated only by statistical analysis of a large body of well-controlled data.

C. Chloroquine. Very little information is available on the effect of chloroquine on pamaquine metabolism. However, no striking elevations of pamaquine plasma concentration have been observed after chloroquine administration.

DISCUSSION

The plasma pamaquine concentration on any given dosage is determined for the most part by localization and metabolic conversion, since its excretion is minimal and absorption is probably rapid and complete.

As evidence for localization, the computation of a "volume of distribution" for a compound such as this, which is subject not only to the process of localization, but also to that of metabolic alteration, is of limited value. The strongest evidence in favor of pamaquine localization in man is that only 6 per cent of the drug can be found in the circulating blood shortly after intravenous infusion. Since neither excretion nor metabolism seems to be rapid enough to dispose of 95 per cent of the drug in the time available, it is reasonable to assume that a large proportion of it has been localized. This is confirmed by the distribution studies in the dog.

The early high peak of 200-400 μ g. per liter in 30-60 minutes after intramuscular injection of pamaquine differs considerably from the lower peaks of 100-200 μ g. per liter in two to three hours after the drug is administered by mouth. This difference is probably ascribable more to the rate of absorption than to any other factor. The variability in mean plasma concentration achieved

within a group of individuals receiving the same dosage of drug is of interest. If this difference were due entirely to variation in the rate of metabolic conversion, it would be expected that the most rapid rates of disappearance of pamaquine from plasma would be found in those individuals who achieved low plasma drug concentrations and, conversely, the slowest rates of disappearance in those who achieved the highest three-hour concentrations. However, on analysis of the disappearance curves of pamaquine from plasma, there was no relation between the height of the peak (or the three-hour concentration) and the rate of disappearance from plasma. Some of those with very high peaks had a rapid rate of disappearance while in some with low peaks the rate of disappearance was quite slow. These observations suggest that localization rather than metabolism is the factor which explains the observed variability.

However, the rapid disappearance of pamaquine from the body is indicative of the importance of the process of metabolic alteration. Study of the pathway of metabolism and of the metabolic products of pamaquine may yield results of practical as well as theoretical interest. In the curative treatment of mosquito-induced vivax malaria (1) no relationship was observed between the mean plasma concentration of the substance measured by the analytical method described, and the clinical effect (cure or relapse). This observation suggests that a metabolic derivative not measured by the reaction with diazotized sulfanilic acid may be the curative agent.

The modification by quinacrine of pamaquine plasma concentration is a striking phenomenon in which both localization and metabolism are probably altered. The very small amount of the acridine capable of excluding pamaquine from sites of localization and/or metabolic channels suggests that quinacrine has a greater affinity for the affected system than has pamaquine.

It has been said for many years that pamaquine toxicity is enhanced by concurrent quinacrine administration. Data presented in another paper (6) support this statement as far as leukopenia and methemoglobinemia are concerned. It is, therefore, proposed that the modification of pamaquine disposition effected by quinacrine represents a specific example of a mechanism whereby one

drug (quinacrine) potentiates the activity (toxic) of another (pamaquine).

Although quinine does not modify pamaquine disposition in as obvious a fashion as does quinacrine, there is little doubt that quinine potentiates the curative action of pamaquine. While neither compound alone is capable of preventing relapses of mosquito-induced vivax malaria with any degree of regularity, a high percentage of cures can be obtained by simultaneous administration of both drugs (1, 7, 8). Moreover, it has been shown that the combination of drugs exerts an effect on hemoglobin metabolism not observed when the drugs are administered separately (9).

CONCLUSIONS

1. Evidence, for the most part indirect, indicates rapid and complete absorption of pamaquine from the gastro-intestinal tract.
2. By direct analysis, in the dog, moderate degrees of localization in liver, lung and brain were demonstrated. Presumably, substantially the same situation obtains in man.
3. Urinary excretion of pamaquine is very small (1 per cent) in the dosage range studied.
4. Disposal of the drug is almost entirely by metabolic alteration. This proceeds at a rapid rate, since eight to 12 hours suffice for the disappearance of 60-mg. doses.
5. The relationship between oral and intramuscular dosage and resulting plasma pamaquine concentration is presented.
6. Quinacrine inhibits the metabolic alteration and possibly the localization of pamaquine.

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

IX. EFFECT OF PAMAQUINE ON THE BLOOD CELLS OF MAN^{1, 2, 3}

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Pamaquine (6-methoxy-8-amino [N-diethyl-aminoisopentyl] quinoline) is recognized to be a potentially dangerous drug. However, a definitive appraisal of its hazard had not been achieved at a time when the further exploration of the antimalarial activity of the 8-aminoquinolines was considered advisable. Pamaquine toxicity can involve the gastro-intestinal tract, the central nervous system, and the circulating blood. Symptoms referable to the gastro-intestinal tract and the central nervous system may be annoying, but there is no evidence that they constitute a hazard to life or persist beyond the termination of therapy. Effects on the blood do constitute a serious hazard and are considered in this paper.

MATERIALS AND METHODS

Most of the patients utilized as subjects either were having malaria at the time of the observations or had had the disease from one to six weeks previously. None of the patients had malaria long enough to be considered likely subjects for the development of blackwater fever. Most subjects were suffering from asymptomatic neurosyphilis and many had received prior antiluetic therapy.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A portion of this work was presented at the meetings of the Federation of the American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 176 and 217.

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⁴ Captain, MC, AUS.

However, whenever indicated, sufficient data were collected in normal subjects.

Plasma pamaquine levels were estimated by the method of Brodie, Taggart and Udenfriend (1). A modification of the method of Horecker and Brackett (2) was utilized for the measurement of methemoglobin.

Special blood studies:

(a) *Isoagglutinins.* Fresh suspensions of group A, B, and O erythrocytes were prepared each test day. The same donors were used throughout the study. The cells were washed once with sterile physiological saline and a 2 per cent solution in saline prepared. Test serum was obtained by allowing the whole blood to clot and centrifuging the sample. Half milliliter samples of the cell suspensions were added to 0.5 ml. of serial dilutions of the test serum in small glass test-tubes. The tubes were placed in a water-bath at 37° C. for three hours. The tubes were then removed, agitated gently, and observed for macroscopic agglutination in a well-lighted, white viewing box. Agglutination was recorded on a scale of 1 to 4 plus. The final titer was taken as the greatest serum dilution that caused agglutination.

(b) *Serum hemolysins.* The tubes containing the group O erythrocytes described in the isoagglutinin technique were examined for the presence of hemolysis.

(c) *Cold hemagglutinins.* Titers of cold hemagglutinins were measured by a technique based on that of Favour (3). Indicator cells were derived from a group O donor whose serum contained no cold hemagglutinins. The titers were recorded as the maximum dilution of saline plus cell suspension at which visible agglutination occurred after 18 hours at 4° C.

(d) *Autoagglutination* was looked for by microscopic examination of a small drop of freshly drawn blood in a large drop of saline. If cold hemagglutinins were present, the slide was kept warm until the examination was completed.

(e) *Fragility of erythrocytes to hypotonic saline.* A 2 per cent suspension of erythrocytes in sterile physiological saline was prepared from blood containing one drop of saturated potassium oxalate per 5 ml. of blood. 0.5 ml. of the cell suspension was added as soon as possible to 2 ml. each of 0.9 per cent sodium chloride (control), to distilled water (total hemoglobin), and to a series of sodium chloride dilutions usually ranging from 0.43 per

cent to 0.30 per cent in steps of 0.02 per cent. The tubes were placed in a water-bath at 37° C. for two hours. The samples were then centrifuged at slow speed for ten minutes. The hemoglobin content of the supernatant fluids was determined on a Coleman Junior Colorimeter. The galvanometer was set at 100 per cent transmission with the supernatant fluid from the 0.9 per cent sodium chloride tube. All readings in each test were made in the same cuvette. The results were expressed in per cent hemolysis at the various saline dilutions uncorrected for the addition of the cell suspension. The highest sodium chloride concentration resulting in 5 per cent hemolysis was arbitrarily chosen as the point of beginning hemolysis.

(f) *The resistance of erythrocytes to mechanical trauma* was measured by the technique of Shen, Castle and Fleming (4).

Section I

METHEMOGLOBINEMIA DUE TO PAMAQUINE

It is well known that methemoglobinemia is a common result of pamaquine therapy (5, 6). The present study is concerned with the degree of methemoglobinemia achieved on various regimens of pamaquine, alone and in combination with quinacrine or quinine. More detailed studies of this phenomenon in relation to blood pigment metabolism will be reported elsewhere.

Observations. Serial methemoglobin determinations were made in patients given various doses of pamaquine, alone or in combination with quinacrine or quinine. These data are summarized in Table I. Methemoglobinemia gradually increases on serial dosage with pamaquine and achieves a fairly stable level by the fifth or sixth day of treatment. For this reason only subjects who had received pamaquine for five or more days are included in the data presented in Table I. The methemoglobins recorded in the table are the maximum values achieved. Although there is

marked variation in methemoglobinemia among individuals on any given regimen, the mean value for each group is proportional to the daily dose of pamaquine, except in the group that also received quinacrine. The mean methemoglobin value achieved by this group on 30 mgm. of pamaquine daily is equal to that of the subjects given 90 mgm. of pamaquine daily but without quinacrine.

Approximately half of each of the first three groups recorded in Table I received 2 grams of quinine daily along with the pamaquine. There was no statistically significant difference between the methemoglobin values achieved by those given quinine and those not given quinine. Very high concentrations of pamaquine, far greater than those achieved in the plasma on therapeutic dosage, are required to produce methemoglobin in erythrocytes *in vitro*. Probably for this reason there was only a rough correlation between methemoglobinemia and plasma drug levels among the patients given pamaquine. However, it was noted that the occasional individual who developed excessively high plasma pamaquine levels on low dosage also had relatively high methemoglobin percentages. Cyanosis was noted in most white individuals who had methemoglobin values greater than 10 per cent.

Weakness and intolerance to exercise were prominent among the patients given 90 mgm. of pamaquine daily, and were also noted among those given 60 mgm. daily. These symptoms were only roughly parallel to the methemoglobin levels and were probably due to some other action of the drug.

Section II

THE PAMAQUINE HEMOLYTIC REACTION

Acute hemolytic anemia constitutes the most serious hazard incidental to the administration of pamaquine. A report of 260 such reactions treated in the Gorgas Hospital in 1942 (7) substantiates this view.

A study of hemolytic anemias occurring among patients given pamaquine was undertaken with the hope that the clarification of the mechanism of their production would be of value in the use of pamaquine or of related synthetic compounds. It was also hoped that the collection of the clinical information necessary for the study of the mech-

TABLE I

Methemoglobin per cent in patients after five to seven days of various pamaquine regimens

Treatment group	Number of subjects	Mean per cent methemoglobin	Standard deviation	Range per cent methemoglobin
Pamaquine, 90 mgm. daily	24	12.2	7.0	0.9-28.7
Pamaquine, 60 mgm. daily	13	8.9	4.5	1.9-20.8
Pamaquine, 30 mgm. daily	29	4.4	2.4	0.7-10.0
Pamaquine, 30 mgm. daily, plus quinacrine	6	12.2	4.6	6.0-19.3

anism of the hemolytic phenomena would yield information on premonitory signs and symptoms of the hemolytic event. Should signs or symptoms be uncovered which would permit the early prediction of the probable occurrence of a hemolytic event, then the termination of pamaquine therapy prior to the appearance of frank hemolysis would constitute a further safeguard during the administration of this agent. Unfortunately, no specific premonitory signs were established.

Such a study is conditioned by the fact that hemolytic anemias are observed in only some 5 to 10 per cent of individuals receiving pamaquine. Such a situation is compatible with the view, that pamaquine per se is a precipitating factor capable of producing an anemia when certain predisposing factors are present rather than the sole etiological agent. Such predisposing factors might be attributable to prior experience with malaria, or some metabolic defect that causes a diversion from the normal in the metabolism of pamaquine. The latter may result in the production in significant amounts of one or more highly noxious agents which in turn are responsible for the hemolytic episode. The studies reported in this section do not settle this problem, although it is apparent that negroes are more prone to develop acute hemolytic anemia than are white subjects. It is possible that prior quinacrine therapy is also a predisposing factor.

Observations. Hemolytic anemia occurred in seven of 157 patients given pamaquine naphthoate in repeated doses for two to 14 days. The race of each patient who developed hemolytic anemia and the preceding events are summarized in Table II.

Five acute hemolytic anemias occurred in negroes and one in a Chinese. A slowly developing hemolytic anemia occurred in a seventh patient who was white and who received 90 mgm. of pamaquine and 2 grams of quinine daily for the treatment of vivax malaria. The plasma pamaquine level and the hemoglobin concentration in relation to pamaquine dosage and onset of symptoms are recorded in Table III. Jaundice, dark urine, or symptoms of acute blood loss were first noted on the third to fifth day of drug administration. A significant loss of hemoglobin was usually noted on the same day as the onset of symptoms. The lowest hemoglobin values occurred one day after the onset of symptoms in four patients, and four and five days after the onset in the other two patients with the acute reaction. Maximal hemoglobin losses varied from 3.7 to 7.9 grams per cent. Serum bilirubin determinations were done during the period of clinical jaundice in three patients. Direct bilirubins were 1.5, 0.5, and 0.8 mgm. per cent, while the indirect values were 3.5, 1.0, and 1.5 mgm. per cent.

Evidence has been adduced (8) to favor the view that concurrent administration of pamaquine

TABLE II
Race and preceding events in hemolytic anemia due to pamaquine

Patient	Race	Species	Duration of parasitemia	Interval between last parasite and pamaquine	Typhoid vaccine infusions	Prior therapy	Interval between end of prior therapy and pamaquine	Fever during pamaquine therapy
			days	days	number		days	
Dal	Negro	<i>P. malariae</i> <i>P. falciparum</i>	14 23	10	8	quinacrine	3	yes
Fra	Negro	<i>P. falciparum</i>	5	0	0	none		yes
Mat	Negro	<i>P. falciparum</i> <i>P. malariae</i>	23 77	6	8	quinacrine	$\frac{1}{2}$	no
Mor	Negro	<i>P. falciparum</i>	31	4	3	quinacrine	0	no
Yat	Negro	<i>P. falciparum</i>	16	7	2	quinacrine	$\frac{1}{2}$	yes
Chi	Chinese	<i>P. vivax</i> <i>P. falciparum</i> <i>P. malariae</i>	10 19 26	4	3	quinacrine	$\frac{1}{2}$	no
Fle	White	<i>P. vivax</i>	4	0	0	quinine	0	no

TABLE III

Hemoglobin loss and onset of symptoms in hemolytic anemias due to pamaquine

The underscored values for hemoglobin indicate the days on which pamaquine was administered.
The day the first symptom was noted is indicated by an asterisk.

Patient	Mean plasma pamaquine ($\mu\text{g./L}$)	Hemoglobin, <i>grams per cent</i> , on various days subsequent to initiation of pamaquine therapy										First symptom	
		1	2	3	4	5	6	7	8	9	12	Type	Day of appearance
Fra	77	13.0	14.3	12.0*	9.8	6.1†	5.4	5.5	5.5†	7.4	8.9	dark urine	3
Yat	217	—	10.6	9.7*	8.3	7.3	8.3	7.3	6.9	7.0	8.2	icterus	3
Dal	high†	—	10.3	9.1	7.4*	5.6†	7.3	6.8	8.8	8.8	9.0	weakness	4
Mor	high	8.6	—	—	—*	2.9	4.8†	6.7	6.5	6.2	8.1	weakness	4
Mat	270	11.3	10.0	10.3	9.1	8.1*	5.9	6.9	5.9	6.2	8.0	icterus	5
Chi	high	—	14.0	14.0	—	7.8*	5.6	—	6.5	6.5	8.8	icterus	5
Fle	201	13.5	13.7	14.8	14.5	12.6	14.0	13.9	12.7	11.9*	10.3	weakness	8

† Level assumed to be greater than 200 $\mu\text{g.}$ per liter since quinacrine present in body.

‡ Transfusion, 500 ml. blood.

and quinine causes some slight destruction of hemoglobin which can be detected only by special techniques. The acute hemolytic process under consideration in this paper is obviously quite different. Furthermore, no significant reduction in hemoglobin content is observed in the blood of patients given pamaquine who do not develop acute hemolytic anemia. For instance, frequent estimations of hemoglobin concentration were made in 54 patients receiving pamaquine following the termination of malaria. The mean hemoglobin concentration of these 54 patients was 11.0 grams per cent before and 11.1 grams per cent after four to six days of pamaquine administration. There were five patients among these who lost one or more grams per cent of hemoglobin during pamaquine administration (1.0, 1.1, 1.5, 1.7, and 2.0 grams per cent). The situation is in contrast to the six patients with acute hemolytic episodes who lost from 3.7 to 9.9 grams per cent of hemoglobin, the onset of these episodes being three to four days after beginning therapy.

Special studies. An examination of factors that might possibly affect the occurrence of hemolytic anemia during pamaquine administration was undertaken. This included consideration of race, dosage, plasma drug level, prior treatment, methemoglobin, agglutinins and hemolysins, and the resistance of the erythrocytes to hypotonic saline and to mechanical trauma.

No hemolytic anemias occurred among 20 pa-

tients (15 negroes, five whites) given 4 to 20 mgm. of pamaquine daily.

Pamaquine was administered in serial dosage in amounts of 30 mgm. or more daily to 157 patients. The incidence of hemolytic anemia during the administration of pamaquine to these patients according to color, prior quinacrine therapy and plasma pamaquine levels is summarized in Table IV. The most definite correlation achieved by these analyses was that between race and occurrence of hemolytic anemia.

It may be noted that all six acute hemolytic anemias occurred among 76 pigmented individuals, while only one subacute anemia was observed

TABLE IV

Incidence of hemolytic anemia due to pamaquine according to race, quinacrine therapy, and plasma pamaquine level

	Pigmented patients		White patients		Total patients	
	Number given pamaquine	Hemolytic anemia	Number given pamaquine	Hemolytic anemia	Number given pamaquine	Hemolytic anemia
Quinacrine	50	5	20	0	70	5
No quinacrine	26	1	61	1*	87	2
High plasma pamaquine	51	5	44	1*	95	6
Low plasma pamaquine	25	1	37	0	62	1
Total	76	6	81	1*	157	7

* Slowly developing hemolytic anemia.

among 81 white subjects. In addition, five of the acute anemias occurred among patients who had received prior quinacrine therapy.

No significant relationship between previous or concurrent malaria fever and the incidence of hemolytic anemia could be established for the group as a whole or among the pigmented patients alone.

Serial methemoglobin determinations were made during the administration of pamaquine to 60 patients. Two of these patients developed acute hemolytic anemia, and one, a slowly developing anemia. The methemoglobin in these patients was 7.2 per cent and 19 per cent before the onset of anemia. However, comparable or greater methemoglobinemia (as high as 28 per cent) was observed among the patients who did not develop a hemolytic anemia.

It was thought that malaria might cause changes in the serum or erythrocytes of certain patients that would predispose the cells to rupture to destruction. If such were the case, then pamaquine or some metabolic product could be considered the precipitating agent in the hemolytic reaction. Only one hemolytic anemia occurred among the patients subjected to these special studies. This precludes a definitive answer to the problem.

The following factors were examined:

(a) *Isoagglutinins*. It has been reported that malaria causes an increase in the titer of isoagglutinins, especially isoagglutinin *a*, and excessively high titers have been reported in two patients with blackwater fever (9). Were this a cause or a predisposing factor in the precipitation of an acute hemolytic anemia, the patient would necessarily belong to Blood Group A or AB. The three patients in the present series who developed an hemolytic anemia during pamaquine administration and who were blood typed, belonged to groups A, A, and AB. However, no isoagglutinins were found in the serum of the group AB patient during the hemolytic episode. In addition, no evidence of increased isoagglutinin titers was found in 17 patients followed serially throughout the course of their malaria (five vivax, 12 falciparum).

(b) *Isohemolysins*. No isohemolysins were demonstrable among the patients described above in section (a).

(c) *Cold hemagglutinins*. Serial examination of the cold hemagglutinin titers was performed in 29 patients with malaria. There was an increase in the titer of seven patients, the maximal titer in six of these without histories of recent respiratory infection being 1 to 40. The seventh patient had a maximum titer during malaria of 1 to 320. However, this patient had recently recovered from an undiagnosed pulmonary infection characterized by slight fever without leucocytosis or cough, and a patch of rales at the right lung base was found on several examinations at the time of the high titer.

Cold hemagglutinins increased to a maximal titer of 1 to 20 in the patient with falciparum malaria who developed hemolytic anemia due to pamaquine administration. However, this increase did not take place until two days after the onset of the anemia. In addition, pamaquine was given to six patients with cold hemagglutinin titers varying from 1 to 20 to 1 to 320 and in none of these was there evidence of an hemolytic anemia.

(d) *Autoagglutinins*. Autoagglutination, in the absence of cold hemagglutinins, was noted in wet drop preparations in three patients. Two patients were receiving pamaquine but no hemolytic anemia developed in either patient. Patient Fra exhibited autoagglutination only after the onset of his hemolytic anemia.

(e) *Fragility of erythrocytes to hypotonic saline*. The resistance of erythrocytes to hypotonic saline was examined serially in 25 attacks of *P. vivax* and *P. falciparum* malaria. The data show a moderate increase in hypotonic saline fragility during the initial days of the parasitemia and then a considerable decrease in fragility some seven to 12 days after the onset of parasitemia. Similar results were obtained in nine patients with active malaria during pamaquine administration. These data will be presented in more detail elsewhere.

(f) *Resistance of erythrocytes to mechanical trauma*. Each "mechanical fragility" (M. F.) was done in duplicate. The mean difference in the per cent hemolysis between the duplicates in 75 tests selected at random is 1.07 ($\sigma = 0.90$). The mean difference between two tests done on different days in 27 subjects during control periods is 1.64 ($\sigma = 1.26$). The mean M.F. in 38 patients (one

to six tests per patient) during control periods is 8.0 ($\sigma = 1.95$) per cent hemolysis.

The effect of malaria on the M.F. of erythrocytes was studied systematically in a series of patients during eight attacks of vivax malaria and 14 attacks of falciparum malaria. The mean M.F. during malarial attacks of varying duration and severity was 8.3 per cent as compared to 8.2 per cent for prior and post malaria control values. No increase in M.F. was noted in patients with malaria which was greater than 2.9 per cent (the mean difference between two consecutive tests in control subjects plus one σ).

The effect of pamaquine alone on the M.F. of 11 patients was also examined. The mean M.F.'s before and during drug administration were 8.4 and 8.8 per cent. There were no individuals with increases greater than 2.9 per cent.

M.F. was studied during the administration of pamaquine to ten patients with active malaria. The mean M.F. of the group during pamaquine administration was 11.4 per cent as compared to the mean control values of 8.0 per cent. In three instances the increases over control values were 6.6, 7.6 and 9.4 per cent, respectively, differences that are statistically significant. The greatest change occurred in a patient who subsequently developed an hemolytic anemia.

However, additional studies on eight patients with vivax malaria who were given large doses of quinine indicate that the M.F. was also significantly increased in this circumstance in four instances. Increased M.F. occurred in three instances when large doses of pamaquine were given to these patients after the malaria was terminated but while quinine therapy was still being given. None of these patients developed hemolytic anemia. These observations indicate that increased M.F. is not useful as a premonitory sign of hemolytic anemia due to pamaquine.

Section III

GRANULOCYTOPENIA DUE TO PAMAQUINE

Hasselmann (10) noted one death due to agranulocytosis and "toxic hepatitis" among 103 subjects given 60 to 120 mgm. pamaquine hydroiodide daily. However, granulocytopenia had not been noted as a consistent manifestation of pamaquine therapy. During the course of studies on the cura-

tive action of pamaquine against vivax malaria, a striking effect on the polymorphonuclear neutrophilic leucocytes was noted. Independently, and at the same time that the present observations were made, Schmidt (11) observed granulocytopenias among monkeys given large doses of pamaquine.

Observations. Eight young adult white volunteers who had been infected by mosquitoes carrying Chesson strain vivax malaria were given 3.0 grams of quinine sulfate over 24 hours, followed by 0.65 gram every eight hours for 15 days, starting on the fourth day of fever. Ninety mgm. pamaquine base daily, in divided doses, were begun two days after the first quinine dose and continued for 14 days.

Daily leucocyte counts were done on each of these eight subjects. One patient, however, is not included in this analysis since pamaquine administration was stopped before the completion of 14 days of therapy. The total daily leucocyte counts for the seven subjects were averaged and presented in Figure 1, in relation to the days of pamaquine administration. This was possible as the curve in each patient was almost identical. A definite rise in leucocyte count occurred in each subject and reached its peak on the sixth or

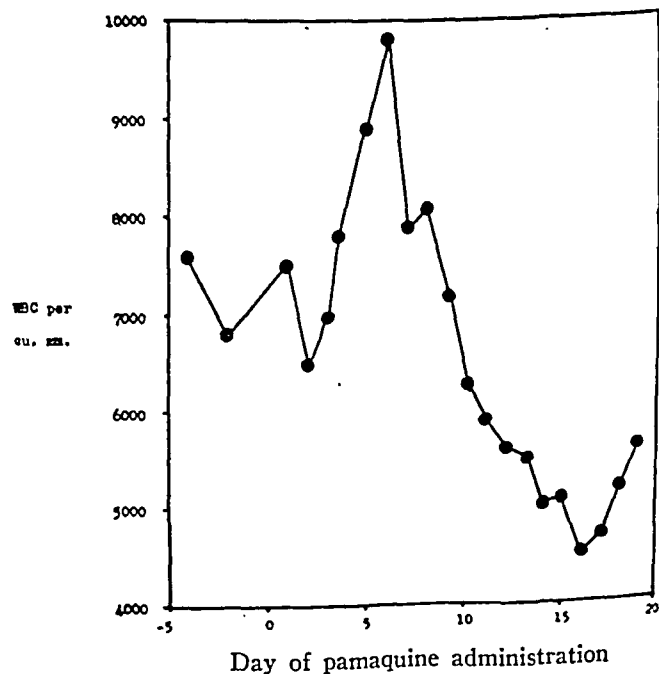


FIG. 1. MEAN WHITE BLOOD CELL COUNTS (TOTAL) IN SEVEN SUBJECTS WHO RECEIVED 90 MG. OF PAMAQUINE BASE DAILY FOR 14 DAYS

TABLE V

Effect on the neutrophilic granulocytes of man of various doses of pamaquine administered for 14 days

Treatment group	Number of subjects	Neutrophilic granulocytes per cubic millimeter			
		Average before pamaquine	Average after 14 days pamaquine	Average decrease	Range of minimum counts
Pamaquine, 90 mgm. daily	12	4762	1714	3048	900-3500
Pamaquine, 60 mgm. daily	4	3878	2920	958	1590-3840
Pamaquine, 30 mgm. daily	11	4650	2890	1950	1600-4600
Pamaquine, 30 mgm. daily, plus quinacrine	6	5093	1493	3600	1150-2100

seventh day of pamaquine administration. There was then a gradual fall in count which reached a minimum 16 to 17 days after beginning pamaquine dosage, or two to three days after stopping the drug. Approximately five days after stopping pamaquine, the leucocytes apparently began a slow return toward normal numbers. The changes in leucocyte counts were entirely due to variations in the numbers of neutrophilic polymorphonuclear leucocytes. No morphological changes were observed in the leucocytes. The remaining neutrophilic polymorphonuclear leucocytes showed a younger distribution than normal. Platelets were not counted, but appeared to be abundant in stained blood films. No sore throats or other infections were noted among the subjects during the period of granulocytopenia. The effect of various dosages of pamaquine is summarized in Table V. Further studies showed that neither concurrent quinine therapy nor a preceding malaria was necessary for the granulocytopenia observed during pamaquine administration, although quinacrine apparently potentiates the effect. Compare groups 3 and 4 in Table V.

GENERAL DISCUSSION

The adverse reactions due to pamaquine which are referable to the blood include the production of methemoglobin, the occurrence of hemolytic anemia, and of neutrophilic granulocytopenia. The occurrence of methemoglobin, per se, is rarely a deterrent to the continued administration of pamaquine since it is unusual, even with high doses, for the oxygen capacity of the blood to be reduced sufficiently to precipitate respiratory diseases. Granulocytopenia represents a potential

danger to life, but this reaction has not been noted at doses ordinarily used in therapy. Furthermore, when it occurs, the process is not precipitous and rapidly disappears on cessation of dosage.

It would appear that the hemolytic reaction is the most seriously toxic hazard of pamaquine. This judgment is based upon the information available in the literature as well as on the experience of this service with the administration of pamaquine under close clinical supervision to some 160 patients over a period of two years.

The mechanisms whereby pamaquine exerts its effects on the formed elements of the blood have not been established by these studies. However, in the instances of the acute hemolytic anemias, it appears that pamaquine or, more likely, one of its metabolic products, acts as a precipitating factor capable of producing hemolysis when certain predisposing factors are present. This hypothesis is based on the observations that acute hemolytic anemia occurs in only a small proportion of individuals given pamaquine, that race has an effect on the incidence of the hemolytic episodes, and that *there is probably no correlation* between plasma pamaquine levels and the hemolytic reaction.

In contrast, methemoglobinemia and granulocytic neutropenia regularly result when pamaquine is given in sufficient dosage, both phenomena are potentiated by quinacrine, which increases plasma pamaquine levels (12), and both phenomena appear to be roughly correlated with plasma pamaquine levels.

SUMMARY

1. The administration of pamaquine results in methemoglobinemia which is roughly proportional to dosage. The effect is potentiated by concurrent quinacrine treatment.

2. Some 5 to 10 per cent of non-Caucasian patients given pamaquine develop acute hemolytic anemia on the third to fifth day of dosage. The incidence of hemolytic anemia among white patients given pamaquine is much lower.

3. The administration of 90 mgm. of pamaquine for 14 days results in a significant reduction of mature neutrophilic granulocytes. Pamaquine in daily doses of 30 mgm. does not have this effect unless quinacrine is also administered.

CASE REPORTS

1. *Acute hemolytic anemia due to pamaquine.* One patient (Fra), a negro, who had not received prior quina-craine therapy, was studied more completely before, during, and after his hemolytic episode than were the others. This patient was born in Puerto Rico, moved to Panama in his youth, and to this country about 15 years ago, but had no history of previous malaria.

During the period of pamaquine administration this patient had active falciparum malaria with considerable fever each day and a parasite density in the peripheral blood as high as 233,000 per cu. mm. Pamaquine dosage was 5 mgm. (free base) every four hours, the mean plasma drug level during therapy being 77 μ g. per liter.

The first clinical sign of hemolysis occurred when the patient voided deep red urine 56 hours after the first dose of pamaquine. The color of the urine during the next several days varied from dark brown to black. Plasma samples obtained during this period were also brown in color. The signs and symptoms observed during the hemolytic episode were indistinguishable from those usually accompanying blackwater fever, although there was no anuria or severe collapse.

The laboratory observations reflected the changes usually associated with acute hemolytic anemia. The hemoglobin fell from an average control value of 13.3 grams per cent to as low as 5.4 grams per cent. The mean corpuscular volume rose during the first day of the reaction but thereafter was normal. The mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were normal throughout. The reticulocytes increased from a control value of 0.2 to 2.1 per cent the day after the reaction and later to 4.5 per cent. The erythrocyte sedimentation rate rose abruptly from 13 to 78 mm. per hour (Westergren). Corrected sedimentation rates (13) indicate that this increase was not due to the anemia alone. There was a leucocytosis of 16,400 per cu. mm. on the second day of the reaction which promptly returned toward normal. There was a definite increase in the "indirect" bilirubin content of the plasma to 3.5 mgm. per cent while the "direct" bilirubin did not change. The resistance of the erythrocytes to hypotonic saline showed a slight increase during the reaction but this was probably due to the malaria and not to the pamaquine or anemia (see above, saline fragility). The erythrocyte mechanical fragility exhibited a marked increase from an average control value of 7.3 per cent to 22.9 per cent. Indeed, the morning before the hemolytic anemia began the mechanical fragility had shown a significant increase to 17 per cent. Increase in mechanical fragility does not occur in malaria alone (see above, mechanical fragility).

The per cent of total hemoglobin present as methemoglobin increased to 7.2, twelve hours before the first evidence of intravascular hemolysis. No methemoglobin could be demonstrated during the next two days, but it reappeared and rose to a value of 10 per cent four days

after pamaquine dosage was stopped. It then gradually disappeared. The pigment responsible for the brown discoloration of the plasma just after the hemolytic episode was methemalbumin (8). Hemoglobin and an unidentified pigment with an absorption band between 475 and 500 μ were found in the black urine voided after the reaction. The urine contained no methemoglobin or methemalbumin.

The patient's blood group was AB (Rh positive, subgroup O) ⁵ and, therefore, there were no isoagglutinins in his serum. There was a slight increase in the cold agglutinin titer after the hemolytic episode. No cold agglutinins were demonstrated before or on the first day of the reaction but the titer was 1:10 on the second and 1:20 on the fifth day after the episode. Autoagglutination was not present before malaria, but the phenomenon was marked the day following the onset of the hemolytic anemia and before the appearance of cold agglutinins. The intensity of the autoagglutination reaction gradually diminished but still persisted in the fourth week of the follow-up period. The autoagglutination and the presence of cold agglutinins made cross-matching for transfusion difficult. However, good cross-matching was obtained using washed red blood cells and the tube technique at 37° C.

Serum hemolysins against compatible group O erythrocytes were not demonstrable. However, incubation of group O erythrocytes for one hour with the patient's serum (obtained the day after the hemolytic reaction) increased the hypotonic saline fragility of the cells to a significantly greater degree than did serum from a normal subject. The erythrocytes showed no tendency to sickle.

The treatment of the anemia in this patient consisted of the oral administration of sodium bicarbonate in large doses to maintain the urine at a high pH and the giving of two blood transfusions. Quina-craine was given by mouth to control the malaria, even though it is known to decrease the rate of pamaquine degradation.

2. *Slowly developing hemolytic anemia.* One of the 12 white subjects given 90 mgm. of pamaquine base and 2 grams of quinine daily during vivax malaria developed evidence of a mild, slowly developing hemolytic anemia. This represents the only hemolytic anemia observed on this service during administration of pamaquine to 81 white subjects. This patient did not develop signs of hemolytic anemia until the seventh day of pamaquine administration and then the hemoglobin fell gradually from 13.9 to 9.7 grams per cent over a period of six days. Prior to the onset of the signs of hemolytic anemia, this patient had a mean plasma pamaquine level of 198 μ g. per liter, methemoglobin rose to 18.7 per cent of the total hemoglobin, and the resistance of the erythrocytes to hypotonic saline showed a slight but significant decrease. There were no significant changes in the erythrocyte mechanical fragility values.

⁵ Rh groupings were kindly performed by Dr. A. S. Williams, Division of Clinical Pathology, Hospital

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STUDIES ON THE CHEMOTHERAPY OF THE HUMAN MALARIAS.

X. THE SUPPRESSIVE ANTIMALARIAL EFFECT OF PALUDRINE^{1, 2}

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Paludrine, N₁-p-chlorophenyl-N₅-isopropylbiguanide acetate, was found by British workers to be the most active antimalarial of a series of synthetic guanidines and biguanidines (1, 2). It possesses high activity against the erythrocytic asexual parasites of both vivax and falciparum malaria and is practically non-toxic at therapeutic doses (3, 4). Previous study of the compound has included extensive prophylactic, curative and suppressive trials by the Australian Malarial Unit at Cairns in experimental sporozoite and trophozoite induced vivax and falciparum malaria (5) and curative trials in naturally acquired relapsing Southwest Pacific vivax malaria (6). The drug has a prophylactic action in falciparum malaria which is complete in single doses as low as 25 mgm. The prophylactic action in vivax malaria is less definite, although the incubation periods of patients in the Cairns studies were systematically prolonged, and subinoculations from infected patients showed that this was due to an effect upon the initial tissue phase (cf. 7) of the disease. It is also effective in the treatment of clinical attacks of malaria, being curative in falciparum but not in vivax malaria. The response of fever and symptoms in each infection is somewhat less prompt

than that obtained with high dosage of quinacrine or chloroquine (8).

The present paper is a report on the assay of the suppressive antimalarial activity of paludrine in standardized blood-induced malarial infections. The studies were designed to yield comparative data on the suppressive antimalarial activity of paludrine and other antimalarials now coming into general use.

MATERIALS AND METHODS

The therapeutic trials were performed in patients⁴ with blood-induced malaria in accordance with standard procedures previously outlined (7, 9). The infections utilized were due to the McCoy and Chesson strains of *P. vivax* and a relatively quinine-resistant strain of *P. falciparum* (Costa). The therapeutic results due to the administration of paludrine are classified in three groups: Class I, no certain effect; Class II, temporary suppression of parasitemia and/or fever; and Class III, a "permanent" effect, i.e., absence of parasitemia for 14 days in McCoy vivax, or 21 days in Chesson vivax and Costa falciparum, followed by a positive reinoculation to demonstrate continuing host-susceptibility to the infection.

Paludrine⁵ was administered by mouth in each instance, and all doses are reported in terms of the free base. Plasma paludrine levels⁶ were estimated by a method that involves coupling of the compound with methyl orange to yield an organic-soluble colored complex (10). The method permits the valid estimation of

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

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⁴ Many of the subjects in this investigation were volunteers from the United States Disciplinary Barracks, Green Haven, New York. The authors express their appreciation to Colonel George Schulz, Lieut. Colonel Nathan Freeman, and Lieut. Colonel Michael D. Buscemi, whose assistance made this work possible.

⁵ Paludrine was generously furnished by The Imperial Chemical Industries, Ltd.

⁶ In many instances, especially, at the lower doses, when only one to four individual doses were used, a stable plasma drug level could not be maintained for the usual four or six days.

plasma paludrine levels as low as 10 $\mu\text{g.}$ per liter. However, considerably lower plasma paludrine concentrations resulted from some of the dosage regimens examined; these were not estimated. A more sensitive chemical method is now available (11).

RESULTS

Paludrine in varying dosage was administered to 28 white subjects with blood-induced McCoy strain vivax malaria (Table I). It was soon apparent that the drug is extremely effective against the erythrocytic phase of this strain of malaria. A permanent eradication of parasites, or Class III effect, was noted in all patients with a four-day mean plasma paludrine concentration of 10 or more $\mu\text{g.}$ per liter. Total doses of paludrine as low as 50 mgm. were sufficient to produce Class III effects with regularity. The lowest total dose investigated, 12.5 mgm., was given to ten patients. Even this minute amount of drug had a dramatic effect on the course of the disease, Class III effects being noted in three instances, while complete

TABLE I

The relationship between dosage and plasma concentration of paludrine and therapeutic effect in four-day tests against blood-induced McCoy vivax malaria

Patient	Total dose	Mean plasma paludrine concentration	Class of therapeutic effect	
			II	III
	mgm.	$\mu\text{g./L.}$		
Bro	225	56		x
Con	225	51		x
Wit	238	48		x
Pow	150	38		x
Tay	150	30		x
Che	150	29		x
Bur	225	23		x
Jor	150	22		x
Sou	150	22		x
Fla	100	21		x
Ham	50	20		x
O'Br	50	17		x
Qui	100	17		x
Mor	50	13		x
Mar	100	10		x
Kli	25	—	x	
Cha	25	—	x	
Ott	25	—	x	
Tha	12.5	—		x
Fit	12.5	—	x	
McL	12.5	—	x	
Dic	12.5	—		x
Pur	12.5	—	x	
Lev	12.5	—	x	
Spe	12.5	—	x	
Mcl	12.5	—		x
Bre	12.5	—	x	
Moo	12.5	—	x	

TABLE II

The relationship between dosage and plasma concentration of paludrine and therapeutic effect in four-day tests against blood-induced Chesson vivax malaria

Patient	Total dose	Mean plasma paludrine concentration	Class of therapeutic effect	
			II	III
	mgm.	$\mu\text{g./L.}$		
Mer	4000	688		x
Mur	4000	464		x
O'Be	500	70		x
Mon	500	59		x
Lyo	500	49		x
Mur	225	40		x
Pet	150	34		x
Pad	150	39		x
Gol	150	28		x
You	225	17		x
Lem	100	—		x
Sca	100	—	x	
Lev	25	—	x	
Sny	25	—		x
Sig	25	—	x	
Smi	25	—		x

temporary, or Class II, effects occurred in the other seven patients.

The effect of paludrine against the Chesson strain of vivax malaria was examined in 16 patients (Table II). This strain had previously been demonstrated to be more resistant than the McCoy strain of vivax to the suppressive antimalarial action of quinine, quinacrine, and chloroquine (7, 12, 13). Total doses of 150 mgm. or more of paludrine resulted in Class III effects. One of two patients given a total dose of 100 mgm. had a Class III effect, the other, a Class II effect. At the lowest total dose studied, 25 mgm., two patients had Class III effects, and two had Class II effects. It is likely that paludrine is only slightly less effective against the Chesson strain than against the McCoy strain.

In contrast, the quinine-resistant Costa strain of falciparum malaria was also relatively resistant to the suppressive action of paludrine (Table III). Total doses ranging from 338 to 750 mgm. were administered to nine patients. The six-day mean plasma paludrine levels varied from 55 to 105 $\mu\text{g.}$ per liter. Class III or permanent effects were noted in two patients with mean plasma drug levels of 78 and 95 $\mu\text{g.}$ per liter, the remainder of the patients having Class II or complete temporary effects, the parasite-free intervals ranging from two to 14 days.

TABLE III

The relationship between dosage and plasma concentration of paludrine and therapeutic effect in six-day tests against blood-induced Costa falciparum malaria

Patient	Total dose	Mean plasma paludrine concentration	Class of therapeutic effect	
			II	III
	mgm.	μg./L		
McC	500	106	x	
O'Ne	750	103	x	
Sca	750	102	x	
Dav	750	95		x
Gib	500	82	x	
Wil	550	78		x
Sha	500	74	x	
Vel	338	68	x	
Ros	500	55	x	

In no instance of treatment with paludrine was an adverse side reaction noted that could be ascribed to the drug.

DISCUSSION

The doses and plasma drug levels of paludrine, quinacrine (12) and chloroquine (13) required to achieve Class III effects in McCoy and Chesson strain vivax malaria and Costa strain falciparum are summarized in Table IV.

It is apparent that paludrine has extraordinary effectiveness against the erythrocytic phase of vivax malaria. The peripheral trophozoites of two strains of vivax can be permanently eradicated with oral doses much smaller than are necessary with other antimalarials such as quinacrine or chloroquine. Permanent eradication of the peripheral trophozoites of Costa falciparum was not regularly achieved at the dosage tested. It should be noted, however, that the dosage used was far

TABLE IV

Total doses and mean plasma drug levels of paludrine, quinacrine and chloroquine required to achieve Class III effects in two strains of vivax malaria and one strain of falciparum malaria

	Paludrine		Quinacrine		Chloroquine	
	Total dose	Plasma level	Total dose	Plasma level	Total dose	Plasma level
	mgm.	μg./L	mgm.	μg./L	mgm.	μg./L
Vivax, McCoy strain	50	10*	700	25*	300	10*
Vivax, Chesson strain	150	17*	1100	25†		
Falciparum, Costa strain	>750	>100†	>1100	65†	650	30†

* 4 day mean plasma drug level.

† 6 day mean plasma drug level.

below the toxic dose (5, 6) and the dose recommended for treatment of clinical attacks of malaria (6).

The study of paludrine was undertaken at too late a date in the malaria program to collect definitive data on the relation between oral dosage, plasma paludrine levels, and antimalarial effects in either vivax or falciparum malarias. As far as falciparum malaria was concerned, there was no apparent correlation between effect and drug levels in the range of 55 to 106 μg. per liter.

The observations upon which these summarizing statements are based do not define the potentialities of paludrine in the routine suppression and treatment of malaria. It seems likely that routine suppression in falciparum malaria is accomplished by a combination of the very high order of the drug's prophylactic action and the moderate order of its suppressive action; whereas, in vivax malaria, it is accomplished by the high order of its suppressive action, perhaps aided by a minor degree of prophylactic action. Routine treatment of the clinical attack will usually involve the administration of high dosage as compared to the dosage utilized in these studies. The effectiveness of such high dosage is best illustrated by the experimental work of others (3, 4, 5, 6).

The high order of antimalarial activity shown by paludrine against more than a single phase of the malarias, *i.e.*, primary tissue phase of falciparum and erythrocytic phases of vivax and falciparum, places the drug in a unique position among the synthetic antimalarials developed in recent years.

SUMMARY AND CONCLUSIONS

1. Paludrine is the most active suppressive agent in vivax malaria yet described, exceeding quinacrine or chloroquine to a considerable extent in this respect.

2. It is less active as a suppressive in falciparum malaria, routine suppression at low dosage being due presumably to its high order of prophylactic action in this infection.

3. Paludrine is apparently non-toxic in therapeutic doses.

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A TECHNIQUE FOR THE DETECTION OF MINIMAL NUMBERS OF MALARIA PARASITES; ITS APPLICATION IN THE DETECTION OF SUPPRESSED VIVAX MALARIA^{1, 2, 3}

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It has been demonstrated, by the subinoculation of large volumes of blood, that malaria parasites are present in the peripheral circulation of individuals who have been bitten by infected mosquitoes but whose disease is suppressed with adequate treatment (1). Since each examination by this procedure requires the use of an additional volunteer subject, a substitute method which yields the same information without necessitating a corresponding utilization of recipients should be highly desirable.

Whereas not less than 10 malaria parasites per mm.³ or 10,000 per cm.³ of blood are required to be uniformly detectable by the usual thick smear examination, the method of Ferrebee and Geiman (2) for the preparation of concentrates of parasitized red blood cells is applicable, with slight modification, to the demonstration of *Plasmodium vivax* at parasite densities far below those required for detection by the routine laboratory method. The modified concentration technique permits the demonstration of parasites when they are present in concentrations of less than 100 per cm.³ of blood.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² A portion of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, March 11-15, 1946, Federation Proc., 1946, 5, 165.

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The present report deals with the application of a concentration procedure to the detection of parasites in patients with suppressed vivax malaria.

The parasite concentration procedure is essentially that of Ferrebee and Geiman (2) modified by a preliminary removal of white blood cells. This additional step is essential since otherwise the white blood cells present in whole blood are concentrated along with the parasitized erythrocytes and the white blood cells may completely obscure the parasites in the final smear.

METHODS AND MATERIAL

Five to 15 ml. of venous blood are drawn into a syringe containing an amount of heparin sufficient to prevent clotting. The blood is transferred to a centrifuge tube and spun for 20 minutes at 2000 r.p.m. The plasma layer is removed, avoiding any disturbance of the buffy coat. The white cells are then aspirated off as completely as possible. The plasma which was removed is then poured back into the tube and the red cells and plasma gently mixed. The reconstituted blood is carefully layered over an equal volume of bovine albumin solution^{5, 6} in a suitable centrifuge tube. The albumin is prepared by dilution⁷ of four parts of the 30 per cent

⁵ The bovine albumin solution used was kindly provided by Dr. Lesh, medical director of the Armour Laboratories, Chicago, Illinois.

⁶ The stock albumin solution should be kept sterile, since yeast cells and bacteria may completely dominate smears prepared with contaminated solutions.

⁷ There is some variation in the dilution of 30 per cent bovine albumin required for best results. This is best assayed for each lot of albumin by making a series of dilutions varying about an average of four parts of albumin to one part of saline and testing them against samples of parasitized blood. Normal blood may also be used for this purpose. In this case, the dilution to be used is selected as the one which, in the final step of the concentration technique, yields a fine layer of red cells just covering the bottom of the centrifuge tube.

bovine albumin solution as furnished, with one part of normal saline solution. The tube is centrifuged at 1000 r.p.m. for five minutes; the speed is then increased to 2500 r.p.m. and continued for 15 minutes. At the end of this time the material has formed three layers. The blood plasma which constitutes the uppermost layer is removed and discarded. The deepest layer is formed by the normal non-parasitized red blood cells which have passed through the middle albumin layer. The latter, which should be a clear yellow or at most slightly tinged with pink, contains the parasitized red blood cells which, because of their diminished density and the relatively high viscosity of the albumin solution, have been left behind. The albumin layer is removed and mixed with two to three volumes of isotonic saline solution in a conical centrifuge tube. The latter is centrifuged at 2500 r.p.m. for five minutes, driving the contained red cells to the bottom where they usually form a layer just sufficient to cover the bottom of the tube. The saline-albumin mixture is carefully aspirated off. The fluid which runs down the sides of the tube is sufficient to suspend the cells which may be taken up in a fine capillary pipette and transferred to a clean microscope slide where the drop is spread to form a thick blood smear of somewhat less than the usual density. When completely dried the smear is stained with Giemsa stain using the technique usually applied to thick blood smears. The parasites do not differ, in their microscopic appearance, from those seen in thick smears prepared without the preliminary concentration.

The individuals utilized in these studies were volunteers⁸ who were inoculated, by the bite of *Anopheles quadrimaculatus* mosquitoes,⁹ with the Chesson strain (3) of *P. vivax*. This strain, originally isolated from a patient who acquired the infection in the Southwest Pacific, is characterized by repeated early relapses occurring at intervals apparently dependent only on the duration of suppressive therapy.

The data on the concentration of vivax parasites are derived from two types of studies. (A) In the first, each individual was inoculated by the bite of infected mosquitoes, all biting being done on a single day and each mosquito being permitted to engorge on the subject bitten. The salivary glands of mosquitoes whose abdomens were found to contain blood were dissected out and examined for the presence of sporozoites. The density of the gland infection was estimated at 0 to 4+ and the inoculum received by each volunteer is given as the sum of the densities of the infections in the mosquitoes by which he was bitten. Drugs administered to this group were given daily in divided doses throughout the period of therapy. Ordinary thick smears were obtained daily and examined for parasites, beginning on the eighth day

after biting and continuing until the development of clinical malaria. Concentrations were done on 10 to 15 ml. of venous blood beginning on the seventh or eighth day after inoculation and continuing until at least two successive positive results were obtained. In five instances, subinoculation of blood to a susceptible recipient was performed on the ninth day. The volume of blood transferred by subinoculation was 200 ml. in three instances but, because no compatible recipients were available, the other two were limited to 10 ml. volumes.

(B) The second type of study was done incidental to the testing of three derivatives of 4-aminoquinoline for their effectiveness as suppressive antimalarials in field-type studies with Chesson strain vivax malaria. The results of this experiment are reported elsewhere (4). The concentrations were done merely to confirm the presence of parasites in the circulating blood and no attempt was made to detect parasites at the earliest possible date. The patients in this study were bitten on three occasions at two-day intervals. The volunteers were divided into ten groups of three. Each subject in a group received a different one of the three drugs. Inoculations were carried out by the interrupted feeding method (5), an equal number of mosquitoes biting each of the three subjects first, second, and third. The drugs were administered in single weekly doses of 0.25 gram of the base, given one week before the first day of inoculation, and on the same day of the two succeeding weeks. Daily examinations for parasites were done by routine smear methods. Concentrations were done at a time during the drug suppression when it was expected that positive results would be obtained.

RESULTS

Preliminary studies with the concentration procedure showed that parasites could be found in the peripheral blood a few hours after the intravenous injection of one million *P. vivax* trophozoites. Blood containing a known number of parasites was diluted serially with normal blood. It was possible to find parasites present in a concentration of 100 per cm.³ but not at 10 per cm.³. Attempts to concentrate the parasites in patients infected with *P. malariae* were unsuccessful as might be expected from the fact that the parasitized cells are not enlarged. The same is true of cells containing the young ring forms of *P. falciparum*. Some success was attained in concentrating the more mature forms of *P. falciparum* that develop when blood containing the young ring forms is incubated for 24 to 48 hours, by a modification of the method of Bass and Johns (6, 7).

The results of the first type (A) of experiment in vivax infections are presented in Table I.

⁸ The volunteers in these studies were inmates of the United States Disciplinary Barracks, Green Haven, New York.

⁹ Many of the infected mosquitoes were furnished by Doctors Clay G. Huff and Frederick Coulston, whose assistance is gratefully acknowledged.

TABLE I

Comparison of concentration with routine smear method in individuals receiving heavy inoculations of *P. vivax* sporozoites on one occasion

Patient	Inoculum*	Last negative concentration	First positive concentration	First thick smear positive
days after inoculation				
No medication				
Mo	47	8	9	10
Pa	47	8	9	11
Oo	35	8	9	10
Pe	35	8	9	12
Na	32	8	9	12
Quinine, 2 grams daily, day 0 to day 13				
Om	68	8	9	22
SN-7618 (chloroquine), 0.3 gram daily, day 0 to day 11				
Ja	44	8	8½	75†
Lo	36	8	8½	70†
Le	35	8	8½	63†
Gr	35	8	8½	81†
Jo	29	8	8½	75†

* Sum of densities of gland infections in mosquitoes biting each subject.

† Subinoculation positive on day 9.

Group I consists of five subjects to whom no suppressive drugs were given. Concentrations were done to find the earliest date on which parasites might be demonstrated and to ascertain the interval by which this would precede detection by routine methods. Parasites are detectable by the concentration procedure on the ninth day after inoculation, preceding detection by the usual methods by one to three days. Group II consists of a single individual who received quinine and five who received SN-7618 (chloroquine) in full therapeutic dosage. Parasites were detectable by the concentration technique 8½ but not eight days after inoculation. In no instance were direct smears of the blood positive during the period of drug administration, or for a considerable time thereafter, the period of negative smears depending on the suppressive drug used.

The results obtained during the field-type suppressive study (B) are presented in Table II. One subject who received SN-6911 is omitted from the table since the tube containing blood for concentration taken on the tenth day was broken

during the procedure. In 28 of the remaining 29 volunteers, parasites were demonstrated by the concentration technique during the period of drug suppression. Of these, five had single positive smears by the routine method during the period of suppression and one had a positive smear on two successive days. The one individual whose blood showed no parasites after concentration failed to develop malaria within 63 days after inoculation at which time observations were discontinued.

TABLE II

Comparison of concentration with routine smear method in field-type suppressive experiment

Patient	Inoculum*			Last negative concentration	First positive concentration	Thick smears positive	
	Day 0	Day 2	Day 4			Transient	Persistent
				days after first inoculation			
SN-6911 (sontochin), 0.25 gram on days -6, 0, 7, 14							
Pa	21	13	8	9	10	—	23
Ni	21	12	9	9	10	14	24
Ha	28	16	7	9	10	12, 13	29
Si	12	15	9	9	10	12	31
Al	10	13	11	14	15	—	28
Ni	14	17	24	—	14	—	28
Pa	7	15	13	—	14	—	29
Ma	15	17	18	—	14	—	26
Br	8	15	13	15	—	—	†
SN-7618 (chloroquine), 0.25 gram on days -6, 0, 7, 14							
Sl	4	7	30	14	15	—	47
Ba	10	14	18	—	14	—	51
Fo	7	17	24	—	14	—	54
Od	6	15	13	—	14	—	42
Gl	16	7	18	—	14	—	49
El	23	7	9	—	9	—	49
Mi	16	12	7	9	10	—	49
Ta	19	12	16	9	10	—	50
Ad	13	13	16	9	10	—	50
Je	22	11	8	—	9	—	48
SN-8137 (oxychloroquine), 0.25 gram on days -6, 0, 7, 14							
Ke	16	0	8	9	10	12	26
Dr	20	5	20	—	9	12	28
Da	9	14	12	9	10	13	26
Co	18	8	8	9	10	—	25
Sm	19	4	15	9	10	—	32
Le	10	10	12	14	15	—	28
Ke	10	20	26	—	14	—	28
Pa	11	14	22	—	14	—	29
Ha	8	13	24	—	14	—	26
Hi	12	17	18	—	14	—	30

* Sum of densities of gland infections in mosquitoes biting each subject.

† Never developed positive smears.

It is probable that the inoculation in this patient was unsatisfactory.

DISCUSSION

Fairley has shown by subinoculation studies (1) that the parasites of *P. vivax* first appear in the circulating blood 8½ days after the bite of infected mosquitoes, and that the time of appearance is not influenced by suppressive drugs. The present studies confirm this finding and demonstrate that it is possible to detect the parasites in the blood of infected individuals just as readily by a simple concentration procedure. These results, furthermore, indicate that even full therapeutic doses of suppressive drugs do not influence the initial out-pouring of the erythrocytic forms of the parasites.

No direct estimation of the sensitivity of the concentration method relative to that of subinoculation is possible from these data. It seems possible that the subinoculation procedure may be the more sensitive method, but other considerations weigh so heavily in favor of the concentration method that the latter would seem to be the more generally useful. Since, at least in heavily infected individuals, parasites can be readily detected by the concentration technique even in the presence of highly effective suppressive drugs, it does possess sufficient sensitivity for most purposes. The concentration procedure is applicable to repeated and frequent studies in the same individual. Results are available within several hours rather than weeks as in the case of subinoculation. And, most important, the application of the concentration method obviates the need for an additional compatible volunteer for each study performed.

The concentration procedure may have some usefulness in the detection of parasites in individuals with fever, suspected of having malaria,

but in whom no parasites can be demonstrated by the usual means. However, since neither *P. malariae* nor the ring forms of *P. falciparum* can be concentrated, the utility of the procedure for this purpose would be limited.

SUMMARY

A technique for the detection of minimal numbers of *P. vivax* parasites in the circulating blood is described.

Application of the procedure to the detection of parasites in individuals with suppressed malaria is described and discussed.

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METHEMALBUMIN. I. APPEARANCE DURING ADMINISTRATION OF PAMAQUINE AND QUININE^{1, 2}

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Hematin was first identified by Schumm (1) as an abnormal constituent of blood plasma occurring in certain hemolytic states. The fact that this pigment does not circulate as free hematin but is combined with serum protein was suspected by Heilmeyer (2) on the basis of a displacement of one of the hematin absorption bands in the presence of serum. The serum albumin-hematin complex was first adequately described and differentiated from methemoglobin by Fairley and Bromfield (3) who studied the pigment in cases of blackwater fever. Fairley (4) named the compound "methaemalbumin" and presented a detailed characterization of its spectrophotometric properties, synthesis and chemical behavior. Spectrograms of the pigment as it occurs in serum of patients with blackwater fever were published by Foy and Kondi (5). Fox (6) based an analytical procedure for the estimation of methemalbumin on the intensity of the absorption band at 623 m μ and its response to certain reagents, sodium cyanide and hydrogen peroxide. The nature of the reaction between hematin and serum albumin was recently examined by J. Keilin (7). The metabolic disposition of methemalbumin is treated in the

paper of Pass, Schwartz and Watson (8) on the fate of intravenously injected hematin.

Methemalbumin has not been demonstrated in normal serum as an intermediary in the metabolism of hemoglobin. It has been observed only as an abnormal serum constituent. In addition to his findings in blackwater fever Fairley (9) demonstrated methemalbuminemia in cases of severe malaria, nocturnal hemoglobinuria, gas bacillus sepsis and pernicious anemia. More recently it has been observed in hemolytic reactions to sulfonamides by Fox and Ottenberg (10) and by Ross and Paegel (11) and in pamaquine hemoglobinuria by Dimson and McMartin (12).

Early in the course of studies conducted at the Goldwater Memorial Hospital on the use of pamaquine in malarial therapy there was encountered an acute hemolytic reaction clinically indistinguishable from blackwater fever. Methemalbumin, in addition to free hemoglobin, was detected in the serum of this patient. This observation directed attention to the abnormal blood pigment.

The antimalarial program included an evaluation of the curative action of pamaquine-quinine therapy. The subjects receiving therapy under this program afforded an opportunity for more detailed study which led to the observation that individuals receiving the combined drug therapy consistently developed methemalbuminemia. Controls receiving either drug alone failed to do so. Whereas in previous studies the pigment appeared in sporadic cases in which the precipitating factors were ill-defined, the present work brings to light the uniform occurrence of the pigment under readily reproducible conditions of drug administration.

¹ This work was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, and Wyeth, Inc., for contributing toward the publication costs.

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TABLE I

Concentration of serum methemalbumin, methemoglobin, hemoglobin, and serum bilirubin during administration of antimalarial drugs

	Patient	Serum methemalbumin* as hemin (mg./L)	Methemoglobin† as per cent of total hemoglobin	Hemoglobin (gm./100 ml.)		Total serum bilirubin (mg./100 ml.)	
				Day of treatment		Day of treatment	
				4	14	4	14
Group I Quinine-Pamaquine	Fl	20	19	14.5	10.0	1.0	2.1
	Bu	22	21	13.0	11.5	1.4	1.3
	Sw	24	20	13.5	12.5	0.9	0.8
	Cl	9	11	14.5	14.0	1.0	1.0
	Mo	4	6	11.0	12.0	1.2	1.0
Group II Pamaquine	We	0	9	11.0	11.5	0.6	0.6
	No	0	1	12.0	10.5	0.6	0.6
	Br	0	13	10.0	12.5	0.7	1.0
	Ed	0	8	12.0	14.5	1.0	1.7
	Go	0	11	12.5	11.0	—	—
Group III Quinine	Ba	0	1	13.5	14.5	1.6	1.3
	Ma	0	1	12.5	12.0	1.6	0.7
	St	0	0.3	10.5	14.5	1.2	0.5
	Ho	0	—	11.5	13.0	1.0	0.5
	Bu	0	0.2	13.5	12.5	0.6	0.6
Group IV Quinacrine-Pamaquine	Do	0	14	10.5	12.5	0.7	0.5
	Br	0	19	12.5	12.0	—	—
	We	0	6	15.0	12.5	0.3	0.4

* Serum methemalbumin measurements were made on the 14th day of combined therapy.

† Methemoglobin concentrations represent maximal values obtained during treatment, usually attained in seven to ten days.

MATERIALS AND METHODS

The experiments were conducted on four groups of white male volunteers infected by mosquito inoculation with *P. vivax* malaria, Chesson strain. Group I, Table I, received combined therapy of 90 mg. pamaquine and 1.8 gm. quinine daily, dosage in each case being expressed as the free base. Group II received 90 mg. pamaquine per day and Group III 1.8 gm. quinine per day. Group IV received combined therapy of 30 mg. pamaquine and 0.25 gm. quinacrine (as base) daily. Drug therapy by oral administration was started on the fifth day of fever and was continued for a two-week period. In the combined treatment quinine or quinacrine was started on the fifth day of fever, pamaquine was added 48 hours later and the two drugs were continued for the two weeks.

Blood specimens were withdrawn prior to therapy for control studies and samples were taken at various times during and after treatment. Fasting serum was employed. To avoid mechanical hemolysis blood was drawn through a No. 18 needle into an oiled syringe containing no anticoagulant. The blood was immediately centrifuged at 2000 r.p.m. for one-half hour. By this procedure a clear layer of serum is obtained separated from the red cells by a deposit of fibrin. Absorption curves in the range of 300 m μ to 650 m μ were measured in the Beckman spectrophotometer, using a cuvette of 10 mm.

light-path. The serum was either undiluted or in the presence of high concentrations of pigment it was diluted three- or five-fold. An aliquot was taken for bilirubin estimation.

The analytical procedure for methemalbumin makes use of the 405 m μ absorption band of the pigment.

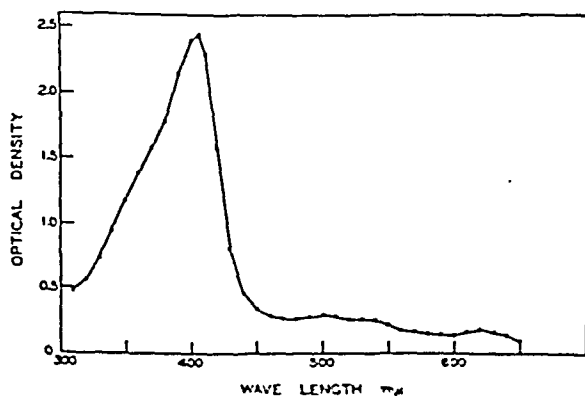


FIG. 1. SPECTROPHOTOMETRIC CURVE OF METHEMALBUMIN FORMED *IN VITRO* BY THE ADDITION OF HEMATIN TO PURIFIED SERUM ALBUMIN

Protein concentration 5 gm. per liter, hemin concentration 20 mg. per liter, pH 7.5.

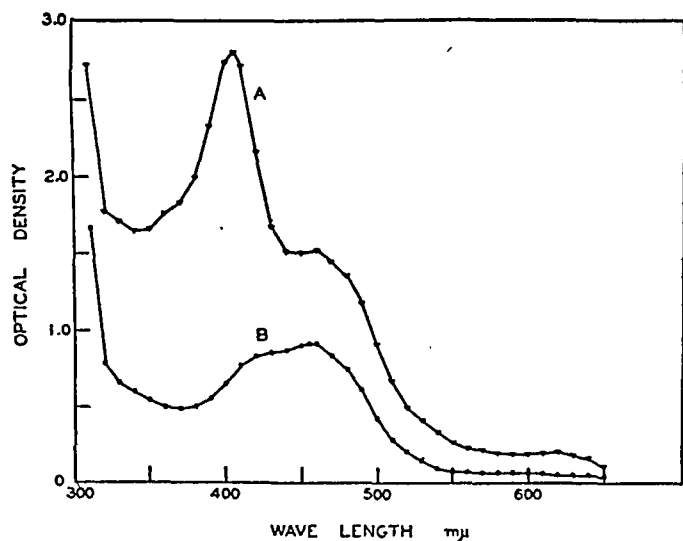


FIG. 2. SPECTROPHOTOMETRIC CURVES ON SERUM OF A PATIENT RECEIVING COMBINED THERAPY WITH PAMAQUINE AND QUININE

Curve A: sample taken after two weeks of daily administration of pamaquine, 90 mg., and quinine, 1.8 gm. Curve B: control sample taken before institution of treatment.

Methemalbumin, Figure 1, exhibits absorption maxima at 405, 500, 540 and 623 $m\mu$. The band at 405 $m\mu$ is the most intense of the series and in this respect is the most suitable for the estimation of small concentrations. The absorption curve of a serum specimen containing methemalbumin is presented in Figure 2, curve A. For comparison there is also included curve B taken on a normal, control sample drawn from the same individual before institution of drug therapy. Although calculation of methemalbumin concentration is based on one point of the curve at 405 $m\mu$, the complete curve provides important information concerning the exact location of the

absorption peaks and also concerning the presence or absence of interfering pigments.

The optical density at 405 $m\mu$ attributable to methemalbumin is computed by subtracting the optical density of normal serum from the measured optical density of the test serum. The serum blank for this computation is taken from Figure 3 which relates the optical density of serum at 405 $m\mu$ to bilirubin content. Independent chemical measurement of bilirubin in each serum under test provides the data necessary for the use of this chart. Additional information regarding the serum blank was obtained from two other sources, namely, measurement of serum taken before the institution of drug therapy, and direct estimation of the serum blank from the shape of the spectrophotometric curve of the test serum.

The optical density data are converted to methemalbumin concentration which is expressed in terms of hemin (ferri protoporphyrin chloride) equivalent. One unit of optical density in a 10 mm. solution depth corresponds to $8.4 \pm .2$ mg. per liter of hemin. This value was derived from experiments on the *in vitro* combination of hematin with normal serum, with purified serum albumin, and with crystalline, human serum albumin.⁴ Figure 4 illustrates such an experiment in which varying amounts of hemin have been added to a constant concentration of purified serum albumin. It is evident that Beer's law is

⁴ We are greatly indebted to Dr. Edwin J. Cohn and Dr. Laurence E. Strong for the supply of purified protein fractions of human plasma and for the sample of crystalline human serum albumin. The materials were prepared in the Department of Physical Chemistry, Harvard Medical School, under contracts recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The products were developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

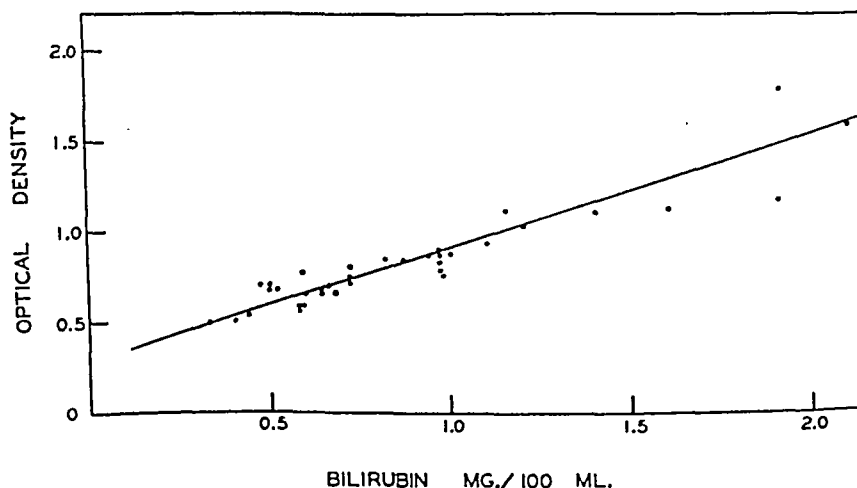


FIG. 3. CHART FOR ESTIMATION OF SERUM BLANK

Optical density at 405 $m\mu$ of human sera containing different concentrations of bilirubin but no methemalbumin.

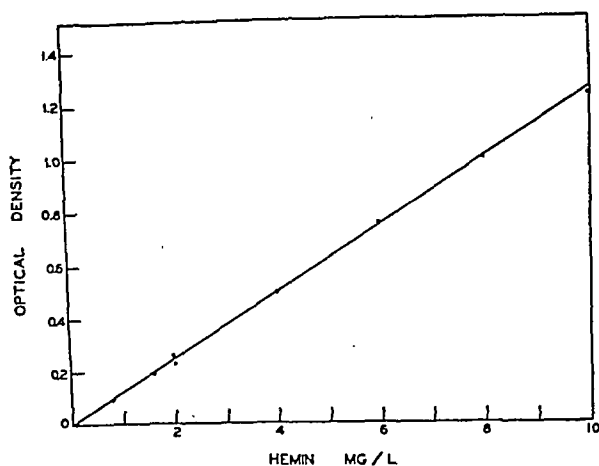


FIG. 4. CALIBRATION CURVE FOR METHEMALBUMIN

Optical density at 405 $m\mu$ of methemalbumin formed *in vitro* by the addition of hemin solutions to human serum albumin, fraction V, run 164. The concentration of protein was 5 gm. per liter. Hemin concentrations were varied up to 10 mg. per liter.

followed closely within the range of measurement. The dissociation constant of methemalbumin is such that possible fluctuations in serum protein concentration do not affect the measurements. Similarly a five-fold dilution of serum for purposes of measurement does not introduce appreciable error. The absorption at 405 $m\mu$ by methemalbumin is independent of pH within the range of pH 7.0 to 9.0.

A typical experiment on the *in vitro* combination of hematin with serum albumin was conducted as follows. Hemin (Eastman Kodak Co.) was recrystallized in the laboratory from pyridine and chloroform according to the procedure of Hans Fischer (13). The human serum albumin was very kindly supplied by Dr. Edwin J. Cohn and Dr. Laurence E. Strong of the Physical Chemistry Department, Harvard University. The samples used in the present study were identified as human serum albumin, fraction V, run 164 and five-times recrystallized human serum albumin, run 179. Serum albumin, 125 mg., was dissolved in 10 ml. of 0.2 M phosphate buffer, pH 7.5. Hemin, 50 mg., was dissolved in 10 ml. of N/10 sodium hydroxide and was made up to 100 ml. with distilled water. From this stock solution of hematin subdivisions were made in N/100 sodium hydroxide, usually starting with a five- or ten-fold dilution. The final test solution was made by adding 2 ml. of the protein solution and 1 ml. of the hematin solution to 2 ml. of 0.2 M phosphate buffer, pH 7.5. The mixtures were held in a water bath for 15 minutes at 38°, then were cooled to room temperature and read in the Beckman spectrophotometer using cells of 10 mm. light-path. The reference solution contained the protein, buffer, and N/100 sodium hydroxide but no hematin. Figure 1 and Figure 4 are based upon data obtained by this procedure.

Although spectrophotometric identification of methemalbumin has been relied on chiefly, the chemical reactions recommended by Fairley (9) have also been applied to identify the pigment. The behavior of the pigment was tested with Stoke's reagent, dilute (10 per cent), and concentrated ammonium sulfide, sodium hydrosulfite, cyanide, and hydrogen peroxide. The differential reactions which identify the pigment were, in general, satisfied though they were not all applied in every case of methemalbuminemia.

No free hemoglobin was encountered in the sera of experimental subjects. If present, oxyhemoglobin could be readily detected spectrophotometrically by the aid of the band at 576 $m\mu$. Oxyhemoglobin has a strong absorption band at 415 $m\mu$ which would seriously complicate measurements on the 405 $m\mu$ band of methemalbumin. Under such circumstances the less sensitive methemalbumin band at 623 $m\mu$ could be used for the estimation.

Serum bilirubin was measured according to Malloy and Evelyn (14). Methemoglobin was estimated by the procedure of Horecker and Brackett (15), total hemoglobin by the method of Evelyn and Malloy (16).

RESULTS

Methemalbumin appeared in the serum of all individuals receiving the combined pamaquine-quinine regimen, Group I. There was considerable variability in the levels attained at the end of the two-week period of therapy, the values ranging between 4 and 24 mg. per liter, Table I.

No methemalbumin appeared in the serum of any individual receiving either pamaquine alone, Group II, quinine alone, Group III, or pamaquine in combination with quinacrine, Group IV. It should be noted that pamaquine dosage was low, 30 mg. per day, in Group IV.

Methemoglobin developed in all patients receiving pamaquine either alone, Group II, in conjunction with quinine, Group I, or in conjunction with quinacrine, Group IV. The methemoglobin concentrations were somewhat higher under the combined therapy than under pamaquine alone. None of the methemoglobin appeared free in the serum; it was confined to red cells. The individuals receiving quinine alone, Group III, showed no significant increment in methemoglobin.

Bilirubin levels were slightly elevated in Group I on combined therapy but were normal in the other three groups when measured at the end of the two-week period of drug administration.

Hemoglobin concentrations dropped during malarial parasitemia but did not appear to be ap-

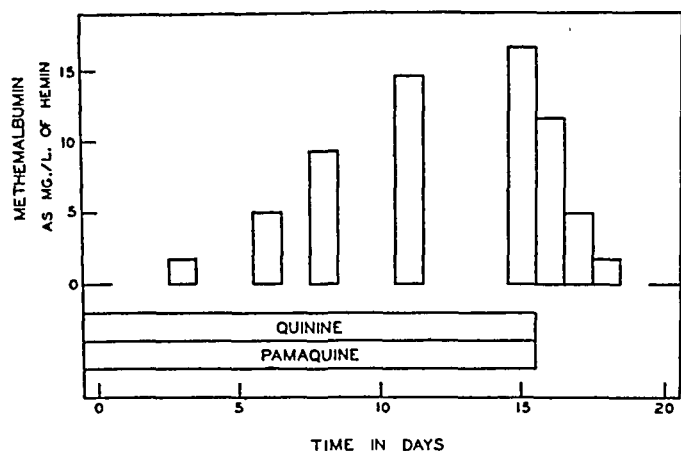


FIG. 5. SERUM METHEMALBUMIN CONCENTRATIONS IN PATIENT J. W. SHOWING ACCUMULATION DURING DRUG ADMINISTRATION AND DISAPPEARANCE FOLLOWING CESSATION OF THERAPY

The drug dosage was pamaquine 90 mg. daily and quinine 1.8 gm. daily.

precipably affected by any of the drug regimens. None of the individuals of the four groups exhibited free hemoglobin either in serum or urine.

The accumulation and disappearance of methemalbumin were followed in one individual by frequent measurements, Figure 5. In this patient the concentration continued to rise throughout the course of drug administration, the rate of rise diminishing toward the end of therapy. The level dropped sharply when drug was discontinued and at 48 hours had fallen to one-third of the peak value. At five days no measurable methemalbumin remained.

The methemalbumin levels of patient J. K. are of particular interest in that she received two

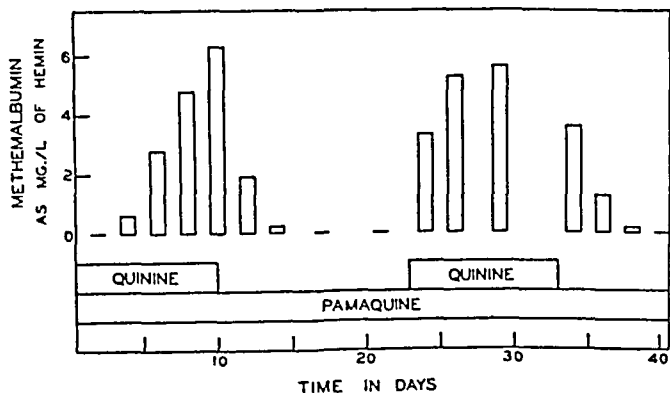


FIG. 6. SERUM METHEMALBUMIN CONCENTRATIONS IN PATIENT J. K. RECEIVING TWO CYCLES OF QUININE ADMINISTRATION (1.8 GM. PER DAY) SUPERIMPOSED UPON CONTINUOUS DOSAGE WITH PAMAQUINE (90 MG. PER DAY)

cycles of quinine administration superimposed upon a continuous pamaquine regimen. As can be seen in Figure 6, methemalbumin appeared only during the periods of combined drug. This patient was a special case to whom the drugs were administered in the absence of malaria.

DISCUSSION

Methemalbumin exhibits characteristic spectrophotometric properties which distinguish it from other blood pigments such as hematin and methemoglobin. The absorption band at 623 $m\mu$ has been utilized in previous work for the identification and estimation of the pigment. In the present study a band at 405 $m\mu$ has been described which, because of its great intensity, permits more sensitive and more quantitative measurements.

In a series of individuals receiving pamaquine and quinine concurrently it has been possible to demonstrate a systematic production of methemalbumin. Such methemalbuminemia was not elicited by the administration of either drug alone or by pamaquine given in conjunction with quinacrine. Production of the pigment in these cases was related to a synergistic combination of the two drugs. It is important to note that the methemalbuminemia developed in the absence of frank hemolysis. The occurrence of the pigment has usually been associated with a massive hemolytic reaction though there have been instances reported, for example in pernicious anemia, where this has not been the case. The relationship of methemoglobin production to the generation of methemalbumin is not clear. Pamaquine alone gave rise to methemoglobin within the red blood cells but caused no methemalbumin to appear in the serum. The combination of pamaquine with quinine enhanced the methemoglobinemia to a moderate degree but this rise in itself certainly could not account for the generation of a totally new pigment, methemalbumin, in the serum.

The antimalarial drugs pamaquine and quinine have long been associated with disturbances in hemoglobin metabolism. Pamaquine therapy consistently gives rise to methemoglobinemia and more rarely in susceptible individuals to acute hemolytic reactions (12). There are a few cases in the literature in which hemoglobinuria has resulted from toxic doses of quinine (17). Quinine

has been incriminated as one of the contributing factors in the pathogenesis of blackwater fever (18).

These reported instances of acute hemolysis are uncommon. They have been detected in the rare individual case by virtue of the spectacular appearance of free hemoglobin in the urine and serum. The regular appearance of methemalbumin in all the individuals receiving combined pamaquine-quinine therapy reveals a more subtle disturbance in pigment metabolism which is consistently evoked by the drug combination.

SUMMARY AND CONCLUSIONS

A procedure is described for the estimation of methemalbumin in serum. The method makes use of an absorption band at 405 m μ and is applicable only in the absence of free hemoglobin.

Methemalbumin consistently appeared in the serum of individuals receiving quinine and pamaquine concurrently. Methemalbuminemia was not elicited by administration of either drug alone or of pamaquine in conjunction with quinacrine.

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METHEMALBUMIN. II. EFFECT OF PAMAQUINE AND QUININE ON PATHWAYS OF HEMOGLOBIN METABOLISM^{1, 2}

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INTRODUCTION

Methemalbuminemia has been described in association with massive intravascular hemolysis (1, 2, 3, 4) or with limited hemolysis in the presence of liver disease (1, 5). Similarly, the injection of large amounts of hemoglobin may result in methemalbuminemia (1), whereas smaller amounts do so only in the presence of a damaged liver (6). Hematin injected intravenously rapidly combines with serum albumin to form methemalbumin (7) which is then converted to bilirubin and is largely recoverable as fecal urobilinogen (8).

It has been noted that the concurrent administration of pamaquine and quinine also leads to methemalbuminemia, whereas neither drug alone produces this effect (9). The reproducibility of the phenomenon suggested the use of these drugs as a means of investigating methemalbumin metabolism. Studies were directed toward the relationship of methemalbuminemia to the degree and duration of hemolysis and hemoglobinemia, to the disappearance rate of methemalbumin as affected by drug action, and to the possible role of liver function.

MATERIALS AND METHODS

White patients with central nervous system syphilis were used as subjects. Some received therapeutic ma-

laria during the study but the period immediately following malaria was avoided because of the questionable status of certain functions of the liver (10, 11, 12). Serum bilirubin and bromsulfalein retention tests were normal, unless specifically mentioned.

Pamaquine naphthoate and quinine sulfate were administered orally, the dosage in each case being expressed in terms of the free base. Plasma pamaquine (13) and quinine concentrations (14) were estimated at intervals to ascertain reliability of drug intake. Pamaquine dosage regimens were either 15 mg. every four hours or 10 mg. every eight hours. Quinine was given as the sulfate in 0.6 gram doses every eight hours.

Commercial hemin⁴ was recrystallized (15) prior to being prepared for intravenous injection (8). One of five patients receiving hematin suffered dizziness and abdominal and back pain of short duration. The injection was discontinued and the patient withdrawn from the study. Uncomplicated thrombophlebitis was encountered following some of the slower injections. No other untoward effects were noted.

Hemoglobin solution⁵ for intravenous injection was received and stored in 500 cc. sterile flasks at a concentration of 7.5 ± 0.5 grams per cent. More than 98 per cent was present as oxyhemoglobin. One of the four patients receiving hemoglobin intravenously experienced abdominal cramps, nausea and vomiting and another, transient back pain. No hyperpyrexia or other untoward effects were encountered.

The estimation of methemalbumin concentration has been described in full in the preceding paper (9). Hemoglobin was estimated by the method of Evelyn (16). Serum hemoglobin was estimated on the Beckman photoelectric spectrophotometer by a method similar to the one used for methemalbumin, except that optical densities were recorded at 415 m μ wave-length, the absorption maximum of oxyhemoglobin. At this wave-length, after the correction blank had been subtracted, an optical density of 0.076 (1 cm. cell) was estimated as equivalent to a hemoglobin concentration of 1.0 mg. per cent.

Serum bilirubin was estimated by the method of Malloy and Evelyn (17), fecal urobilinogen by a modification of Watson's method (18) yielding 90-100 per cent recovery, coproporphyrin by the method of Dobriner and Rhoads (19), and bromsulfalein retention in per cent

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

² The authors express their thanks to the Malaria Study Section of the National Institute of Health for editorial assistance and for arrangements in regard to the publication of this paper. They are also grateful to the Abbott Laboratories, E. I. du Pont de Nemours and Company, Inc., E. R. Squibb and Sons, Eli Lilly and Company, Sharp and Dohme, and Wyeth, Inc., for contributing toward the publication costs.

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⁴ Eastman Kodak Company.

⁵ Supplied through the courtesy of Dr. Robert B. Pennell of Sharp & Dohme, Inc.

by the estimation of serum bromsulfalein at five and 30 minutes after the intravenous injection of 5 mg. per kilo of body weight.

RESULTS

Hemolysis during combined pamaquine-quinine administration. Fecal urobilinogen excretion was estimated in two individuals during successive

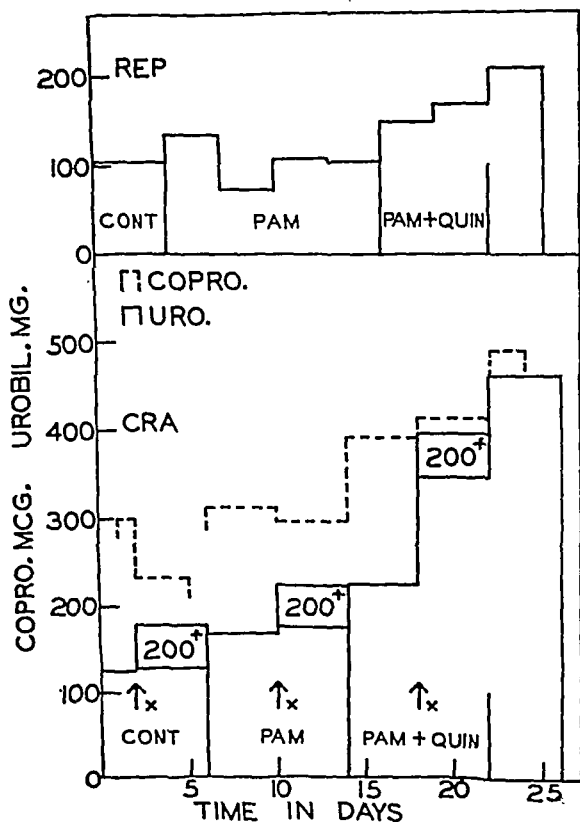


FIG. 1. FECAL UROBILINOGEN AND TOTAL COPROPORPHYRIN EXCRETION AS AFFECTED BY THE ADMINISTRATION OF PAMAQUINE AND COMBINED PAMAQUINE AND QUININE

The excretion per 24 hours of the two pigments was estimated during a control period (CONT), during the administration of 90 mg. of pamaquine base daily (PAM), and during the administration of 90 mg. of pamaquine base and 1.8 gm. quinine base daily (PAM + QUIN). 48-96 hour stool specimens were pooled as indicated in the figure.

The excretion on patient Cra was complicated by the intravenous administration of 400 mg. of hematin, as indicated by the arrows on the lower part of the figure. The four-day period following each hematin injection includes a blocked off area which represents the urobilinogen excretion from 50 per cent of the injected hematin on the assumption that a minimum of 50 per cent is recoverable as fecal urobilinogen (8).

control, pamaquine, and combined pamaquine-quinine periods (Figure 1). Pamaquine dosage was 90 mg. per day throughout in both patients. Only during combined drug administration was urobilinogen output significantly increased over the control period. A slightly increased output of doubtful significance occurred during pamaquine alone in one patient. Total coproporphyrin excretion, estimated in one of the patients, also showed an increased output only during combined pamaquine-quinine administration (Figure 1). The administration of 400 mg. of hematin had no effect on coproporphyrin output in this individual.

Methemalbuminemia not a result of hemolysis alone. One of the patients mentioned above developed methemalbuminemia on the third day of combined drug administration. It was estimated from the increment in fecal urobilinogen excretion that no more than 2.5 grams of hemoglobin was catabolized per day in excess of the patient's control rate. A comparable amount of hemoglobin was administered intravenously to five patients who were receiving no drug to determine whether an equivalent degree of hemolysis alone would produce methemalbumin. Two patients received, respectively, single doses of 7.0 and 9.0 grams of hemoglobin in one hour (Table I) and three received multiple small injections of 4.5 - 6.5 grams per day in divided doses for periods of two to four days (Figure 2). None developed methemalbuminemia. One of the latter patients subsequently was given a large single dose of 15.0 grams of hemoglobin. Methemalbumin was present in the serum of this patient at 24 hours, at which time no free hemoglobin was demonstrable (Table I).

Methemalbuminemia following hemoglobin injection during pamaquine and/or quinine administration. Two patients were given intravenously 4.5 - 6.5 grams of hemoglobin a day in divided doses for three days during pamaquine and for three days during quinine administration (Figure 2), to ascertain whether either drug alone would promote methemalbumin formation if free hemoglobin were available. No significant methemalbumin appeared in one patient during the pamaquine period, whereas in the other, there was trace formation. Quinine caused clear-cut methemalbumin formation under the same conditions of hemoglobin administration.

TABLE I

Serum optical density at 405 and 415 m μ wave-lengths (the absorption maxima of methemalbumin and hemoglobin respectively) 24 hours after a single intravenous dose of hemoglobin

Hemolysis resulting from the handling of the blood or hemoglobinemia is judged qualitatively by the shape of the absorption curve from 540 m μ to 600 m μ wave-length.

Patient	Hemo- globin dose	Serum bilirubin	Optical density 405 m μ			Optical density 415 m μ			Hemolysis	Methem- albumin
			Actual	Expected	Difference	Actual	Expected	Difference		
ROF	7.0	.42	.564	.565	-.001	.619	.595	+.024	0	0
KEN	9.0	.45	.669	.580	+.089	.707	.615	+.092	+	0
BRA	15.0	.47	1.860	.590	+1.270	1.683	.630	+1.053	0	+++
BRA (at 48 hours)		.40	.905	.550	+.355	.895	.585	+.310	trace	++

Two patients received a similar series of hemoglobin injections during combined pamaquine-quinine therapy (Figure 2). Additional methemalbumin was formed from the hemoglobin in-

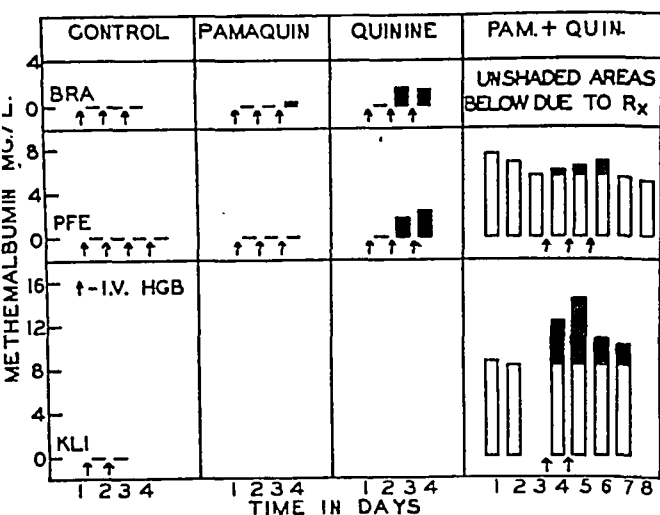


FIG. 2. METHEMALBUMINEMIA AS OBSERVED DURING THE INTRAVENOUS ADMINISTRATION OF MULTIPLE SMALL DOSES OF HEMOGLOBIN

Hemoglobin was administered in daily dosage of 4.8 grams (BRA), 5.0 grams (PFE), or 6.5 grams (KLI) on successive days as indicated by the arrows in the figure and was given during control periods and during the administration of 90 mg. pamaquine base daily and/or 1.8 grams quinine base daily. Rest periods of at least four days were allowed between each course of drug administration. The dosage of pamaquine during combined drug administration was lowered in patients PFE and KLI from 90 to 30 mg. daily after four days at the higher dose. This was done in order to obtain a stable or falling methemalbumin level prior to hemoglobin injection. The unshaded areas during combined drug administration represent methemalbumin attributable to therapy alone; the shaded areas, that resulting from hemoglobin injection. Such a division is at best an approximation.

jected and was superimposed upon pre-existing methemalbuminemia present as the result of pamaquine-quinine administration. The amount of additional methemalbumin derived from the intravenously injected hemoglobin was of the same order of magnitude as that derived from hemoglobin during the administration of quinine alone.

Disappearance rate of serum hemoglobin related to combined pamaquine-quinine administration. The above observations suggest that quinine

TABLE II

Disappearance rate of hemoglobin injected intravenously over a period of one hour during control and combined pamaquine-quinine administration periods

Patient ROF received 7.0 grams of hemoglobin on both occasions, patient KEN 9.0 grams on both occasions. Pamaquine dosage was 15 mg. every six hours and quinine dosage 0.3 grams every six hours for both patients.

Time (hours)	Serum hemoglobin concentration (mg. per cent)		Diminution per hour			
			Concentration (mg. per cent)		Per cent	
	Control period	Drug period	Control period	Drug period	Control period	Drug period
Patient ROF						
2	197	268				
4	107	111*	45	79	23	30
7	26	18*	27	31	25	28
Patient KEN						
2	240	245				
4	108	103*	66	71	28	29
7	19	21*	30	27	28	26

* Methemalbumin formation makes these values somewhat inaccurate, more particularly at the seven-hour period.

and combined pamaquine-quinine interfere in some way with the metabolism of injected hemoglobin and so promote methemalbumin formation. For this reason, the disappearance rate of hemoglobin from serum was estimated in two patients during control and combined drug regimen periods (Table II). Combined pamaquine-quinine caused no demonstrable alteration in the disappearance rate of injected hemoglobin. The methods used for the estimation of serum hemoglobin concentration are subject to some error during combined drug at the four-hour and, more particularly, at the seven-hour periods due to the appearance of methemoglobin and methemalbumin. However, it was determinable that no marked acceleration or retardation of hemoglobin removal occurred.

Methemalbumin disappearance rate as affected by drug administration. On the assumption that one of the drug regimens might interfere with methemalbumin degradation and thereby lead to its accumulation in the serum, methemalbumin disappearance rates were estimated in four patients. 400 mg. of hematin were injected intravenously prior to each determination in order to achieve a high initial level of methemalbumin. Three in-

TABLE III

Change in serum methemalbumin concentration (expressed in mg./L as hemin) following the intravenous injection of hematin, as influenced by drug administration

Pamaquine dosage was 15 mg. of the base every four hours and quinine dosage was 0.6 gram of the base every eight hours.

	Diminution in methemalbumin concentration per hour (mg./L as hemin)				
	Interval				
	1-4 hours	4-12 hours	12-24 hours	24-48 hours	48-72 hours
Patient CRA					
Control	5.7	3.6	1.6	0.75	0.27
Pamaquine	4.1	3.4	1.8	0.72	0.39
Pamaquine-quinine*	3.9	3.3	1.5	0.87	0.31
Per cent diminution per hour					
Control	6.4	5.0	3.4	2.7	2.7
Pamaquine	4.2	4.0	3.2	2.1	2.1
Pamaquine-quinine*	4.3	4.2	2.8	2.4	2.1

* See footnote 6.

TABLE IV

The disappearance rate of serum methemalbumin following the intravenous injection of 400 mg. of hematin as influenced by drug administration

Pamaquine and quinine dosages were the same as those listed in Table III. The probability figures indicate that the slopes obtained during drug administration are significantly different from the control on the same individual.

Pa-tient	Regimen	Methemalbumin concentration (mg./L)			Average diminution per 24 hours (per cent)	Slope	Probability
		24 hours	48 hours	72 hours			
CRA	Control	27.9	9.94	3.61	64.1	-.444	
	Pamaquine	35.4	18.0	9.11	49.3	-.302	<.01
	Pamaquine-quinine*	37.2	16.6	8.44	52.3	-.332	<.02
WIT	Control	31.7	7.77	2.97	72.7	-.586	
	Pamaquine-quinine*	21.7	7.65	3.02	62.7	-.428	<.01
REP	Control	27.3	6.94	1.64	75.5	-.611	
	Pamaquine	25.9	8.59	2.73	67.5	-.488	<.02
MAS	Control	42.7	20.0	7.62	57.6	-.374	
	Control	39.9	18.6	9.03	52.9	-.322	>.18

* See footnote 6.

jections were given eight days apart to the first patient. The first was given during the control period, the second during pamaquine administration, and the third, during combined pamaquine-quinine administration. The concentration of methemalbumin was estimated at intervals and the per cent diminution per hour calculated for specified periods (Table III). The rate of disappearance under all conditions diminished during the first 24 hours, after which it remained essentially constant.⁶ Pamaquine and combined pamaquine-quinine appear to have lowered the disappearance rate. This effect is more apparent during the first 12 hours, but the order of magnitude is sufficiently low to be of uncertain significance. Comparison of the disappearance rates in all patients was made on the basis of the methemalbumin concentrations estimated at 24, 48, and 72 hours (Table IV). The calculated average rates of methemalbumin removal during pamaquine and during combined drug administration were

⁶ To calculate the disappearance rates during combined pamaquine-quinine administration, the methemalbumin already present as a result of drug therapy was subtracted from the total value obtained. This correction is admittedly only an approximation.

each about 11 per cent lower than during the control periods. Apparently quinine, when combined with pamaquine, did not augment the retardation due to pamaquine alone.

The data on serum bilirubin formation and disappearance following the intravenous administration of hemoglobin and hematin during pamaquine suggest some similarity to liver disease. The serum bilirubin concentration curves during pamaquine or combined pamaquine-quinine in these situations were more elevated than during the control period on the same individual (Figure 3).

DISCUSSION

The methemalbuminemia which is observed during the concurrent administration of pamaquine and quinine appears to be the result of two phenomena, viz., an increase in the catabolism of hemoglobin and an interference in its subsequent degradation. The experimental data indicate that the first of these two phenomena results from the synergistic action of pamaquine and quinine, whereas the second may be due to the action of quinine alone. This viewpoint is justified by the following observations. Increased hemoglobin catabolism as evidenced by increased fecal urobilinogen excretion is apparent during combined drug administration but not when either drug is administered in the same dosage alone.⁷ When moderate hemolysis is simulated by small intravenous doses of hemoglobin, no measurable methemalbumin is formed. During quinine administration, such simulated hemolysis does lead to the definite formation of serum methemalbumin. Such methemalbumin formation is of the same order of magnitude as when similar injections of hemoglobin are given to patients on a combined drug regimen.

During the administration of pamaquine alone, simulated hemolysis may lead to trace formation of methemalbumin and, when added to quinine, pamaquine may slightly increase the methemalbuminemia resulting from the intravenous injections of hemoglobin. This effect of pamaquine is presumably due to a retardation of the removal of

serum methemalbumin rather than an increase in its formation, as is the case with quinine. This assumption seems justified by the data which indicate that pamaquine retards methemalbumin disappearance by about 11 per cent per day, an effect not augmented by quinine.

Further observations on the action of quinine and pamaquine are less conclusive. Combined pamaquine-quinine does not significantly alter the disappearance rate of injected hemoglobin. This observation excludes retention of serum hemoglobin as the cause of methemalbumin formation. It does not exclude the possibility that an increased percentage of the hemoglobin that breaks

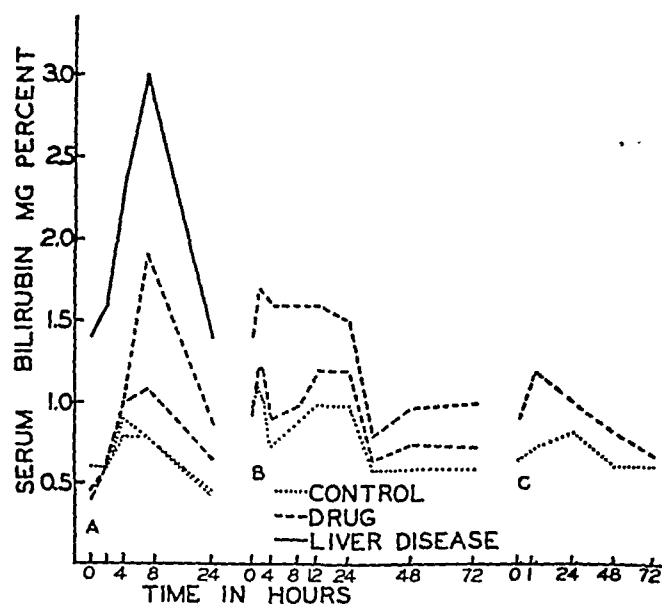


FIG. 3. SERUM BILIRUBIN CONCENTRATION CURVE FOLLOWING THE INTRAVENOUS INJECTION OF HEMOGLOBIN OR HEMATIN AS INFLUENCED BY DRUG ADMINISTRATION OR LIVER DISEASE

(A) Two patients received hemoglobin intravenously, one 7.0 grams, the other, 9.0 grams, during a control period (dotted line) and during combined pamaquine-quinine administration (dashed line). One patient with well documented cirrhosis of the liver received 11.7 grams of hemoglobin (solid line).

(B) Patient CRA received 400 mg. hematin intravenously during a control period (dotted line), during pamaquine administration (lower dashed line), and during combined pamaquine-quinine administration (upper dashed line).

(C) Patient REP received 400 mg. hematin during a control period (dotted line) and during pamaquine administration (dashed line).

Pamaquine dosage was 60 mg. base daily for the patients in (A), 90 mg. base daily for those in (B) and (C).

⁷ It has been assumed that quinine alone in the doses given does not elevate fecal urobilinogen excretion. Unpublished data on one patient are consistent with this assumption.

down is converted to hematin rather than some other pigment.

Patients receiving combined pamaquine-quinine for a week or more develop significant hyperbilirubinemia (9). This is most likely a reflection of impaired liver function in the presence of added hemoglobin destruction since greater increases in fecal urobilinogen than those found, occur without significant hyperbilirubinemia (20). In addition, pamaquine causes abnormal elevation of serum bilirubin concentration following the intravenous injection of large doses of hemoglobin or hematin. It is also probable that pamaquine may retard the removal of methemalbumin from serum indirectly by impairing some functional capacity of the liver. Similarly, one may speculate that liver disease causes some degree of methemalbumin retention.

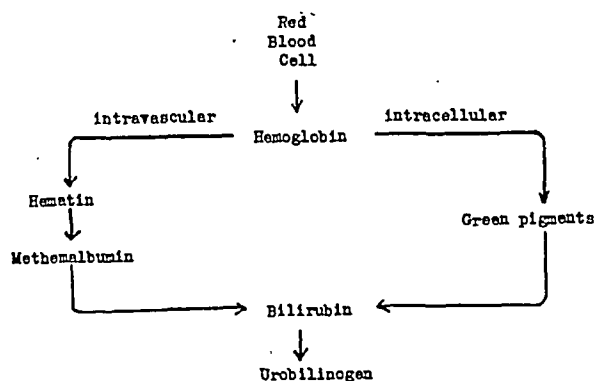


FIG. 4. A DIAGRAMMATIC REPRESENTATION OF THE METABOLISM OF HEMOGLOBIN LIBERATED INTRAVASCULARLY

The formation of the green pigments presumably takes place in the reticulo-endothelial system.

Normally, hemoglobin catabolism is an intracellular process and presumably little or no serum methemalbumin is formed (21). Figure 4 is a diagrammatic representation of one metabolic scheme of intravascular hemoglobin breakdown, including methemalbumin in its probable relation to the other pigments under discussion. When, as in hemolytic diseases, hemoglobin is liberated into the bloodstream, it may be degraded to hematin as well as to the green pigments. Duesberg (21) considered small amounts of injected hemoglobin to be almost exclusively converted to bilirubin by the normal pathway, methemalbumin occurring only under abnormal conditions. Ross (3, 4) has

concluded that methemalbuminemia following massive intravascular hemolysis is a reflection of the retention of serum hemoglobin due to saturation of the normal paths of hemoglobin removal.

Conversely, Fairley (1) has suggested that regardless of the degree of hemolysis some of the intravascular hemoglobin is converted to methemalbumin. He observed that the intravenous injection of large amounts of hemoglobin, 14 grams or more, produced measurable methemalbumin formation, an effect not produced by smaller amounts. This has been confirmed in the present study (Table I), the methemalbuminemia 24 hours after 15 grams of intravenous hemoglobin resulting without associated hemoglobin retention. The disappearance of methemalbumin appears to be slower than normal during pamaquine administration and is probably also slowed by liver disease (see previous discussion). In both conditions methemalbumin may be observed in the serum following the injection of hemoglobin otherwise too small in quantity to cause measurable methemalbumin formation (6). These observations lend support to the hypothesis that even small amounts of intravascular hemoglobin normally break down to form methemalbumin, which becomes apparent only if its removal from the serum is retarded. For these reasons methemalbuminemia is believed to be a reflection primarily of the quantity of free hemoglobin in serum rather than the retention thereof.

The fate of methemalbumin has been well established by Pass, Schwartz and Watson (8) who demonstrated that injected hematin is quantitatively converted to bilirubin. Consistent with this are the observations made in one patient on two occasions. Following the intravenous injection of hematin fecal urobilinogen excretion is increased and total coproporphyrin excretion is not appreciably changed.

SUMMARY AND CONCLUSIONS

The drugs pamaquine and quinine act synergistically to cause increased hemoglobin catabolism. An amount of hemoglobin equivalent to the excess catabolized by combined pamaquine-quinine, injected intravenously, does not result in measurable methemalbumin formation in normal individuals.

During quinine and, to a much lesser extent, during pamaquine administration, the intravenous injection of hemoglobin does result in methemalbuminemia. Quinine acts independently in some way to increase the amount of intravascular hemoglobin degraded to hematin. Pamaquine acts independently to retard slightly conversion of methemalbumin to bilirubin, possibly indirectly, by impairing some functional capacity of the liver. In this manner, pamaquine is believed to cause methemalbumin to accumulate to a measurable quantity during intravenous hemoglobin administration.

Methemalbumin is considered a normal intermediary in hemoglobin metabolism whenever free hemoglobin is present in the serum. Methemalbuminemia becomes apparent only when hemolysis or simulated hemolysis releases a large quantity of hemoglobin into the plasma (*i.e.*, about 10–15 grams), or with lesser amounts when the conversion of methemalbumin to bilirubin is retarded, allowing the former to accumulate in the serum.

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MINERAL BALANCE DURING BRIEF STARVATION. THE EFFECT ON SERUM ELECTROLYTES AND MINERAL BALANCE OF MAINTAINING THE INTAKE OF CERTAIN MINERAL CONSTITUENTS¹

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It has long been known that, during starvation, negative nitrogen balance is accompanied by losses of the chief mineral constituents of both the extra- and intracellular compartments (1, 2). In prolonged fasting (1), in brief fasts followed by graduated increased feeding, and in graduated reduced feeding periods (3), nitrogen, potassium and phosphorus are lost in amounts closely approximating their relative concentration in normal muscle protoplasm, suggesting an even and proportionate expenditure of these intracellular constituents. It was our objective to determine what would occur over a brief period of complete fasting if certain of the usual electrolytes of the diet were furnished orally in the total absence of calorogenic material.

Two obese male patients were studied; one suffered with diabetes but of such mild intensity that, with the dietary restrictions imposed, he was normoglycemic and aglycosuric. Each patient was observed during two periods of total starvation for four days each, with an intervening period of six days during which he was fed a 1200 calorie diet. In one starvation period water only was allowed, in the other starvation period the same amount of water, plus the sodium, potassium, chloride and phosphorus of the 1200 calorie diet, was administered. The behavior of the weight, electrolyte and nitrogen balances, and changes in certain blood constituents were noted.

Case I

B. S. (J. H. H. No. 402163), a 53-year-old South American business man, consulted us because of obesity. His family history did not contain relevant disease. He had enjoyed excellent symptomatic health. Varicose veins

¹ The work described in this paper was carried out under a contract between the Johns Hopkins University and the Office of Naval Research.

of the lower extremities, with slight to moderate afternoon ankle edema, had been present for 10 years. At the age of 40 the patient had weighed 90 kilos. His weight had progressively increased, and at the time he came under our observation, was 128.7 kilos. Four years prior to admission, polyuria and polydipsia had developed, and glycosuria was discovered. He had used insulin for awhile, but his carbohydrate tolerance was such that he was entirely aglycosuric on a diet of 1200 calories.

Examination showed nothing abnormal other than tremendous obesity which was generalized and symmetrically distributed, and varices of the lower extremities, with slight pitting edema. His height was 168 cm. and his weight 128.7 kilos. Blood pressure was 115/70. Blood morphology was normal. Urine, with specific gravity of 1.030, contained neither sugar nor albumin; and its sediment was clear. Phenolsulphonphthalein excretion was 60 per cent in the first half hour, 85 per cent in two hours. There were no abnormalities in the electrocardiogram. Circulation time was 17 seconds with calcium gluconate; venous pressure was normal, and vital capacity was 3.5 litres.

During the experimental period of 14 days, the patient's activity was standardized. He remained at bed rest with the exception of a 30-minute period each day allowed for shaving and washing. At no time during his stay did the patient have glycosuria, and his highest fasting blood sugar was 124 mgm. per cent. Observations were divided into four periods as follows:

Foreperiod. For four days the patient was given a diet calculated to yield 1200 calories, divided into thirds, with 100 grams of carbohydrate, 80 grams of protein, 53 grams of fat. Balance data were not obtained during this period, and the diet was not analyzed. Water was allowed *ad libitum*. His weight fell from 128.7 kilos to 125.2 kilos. The slight edema disappeared.

Period I. This period lasted four days. During it the patient received no food but was given a mixture of $\text{Ca}_3(\text{PO}_4)_2$, KCl, NaCl, and NaHCO_3 in 800 cc. water, taken in divided doses at 8 a.m., 12 noon, 4 p.m. and 8 p.m. By analysis this mixture contained 109 meq. Na; 60 meq. K; 122 meq. Cl; 3400 mgm. Ca, and 1490 mgm. P.² Total water intake was maintained at 3000 cc. per day.

² The salt mixture was designed to approximate the amount of sodium, potassium, chloride and phosphorus

TABLE I
Analytic data

Per.	Date	Wt.	Cal.	Intake						Urine Vol.	Urine						
				N	Na	K	Cl	Ca	P		N	Na	K	Cl	Ca	P	Ti- trable acidity
		<i>kilos</i>		<i>gm.</i>	<i>meq.</i>	<i>meq.</i>	<i>meq.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>cc.</i>	<i>gm.</i>	<i>meq.</i>	<i>meq.</i>	<i>meq.</i>	<i>mgm.</i>	<i>mgm.</i>	
I	Oct. 23-24	125.2	0	0	109	60	122	3400	1460	1440	9.2	123	72	117	152	822	37.2
	24-25	125.0	0	0	109	60	122	3400	1460	2060	12.6	153	94	180	319	1135	49.8
	25-26	124.0	0	0	109	60	122	3400	1460	2680	10.9	155	97	168	394	1270	70.1
	26-27	122.7	0	0	109	60	122	3400	1460	2710	9.5	177	95	144	380	1290	75.5
II	27-28	121.2	1200	13.5	118	74	90	1148	1562	1740	14.9	42	94	64	308	1640	113.0
	28-29	121.6	1200	13.5	118	74	90	1148	1562	1260	11.1	14	48	51	174	840	39.6
	29-30	122.1	1200	13.5	118	74	90	1148	1562	1160	8.3	12	39	36	155	615	26.6
	Oct. 30-31	122.2	1200	13.5	118	74	90	1148	1562	990	9.5	26	37	44	193	770	35.8
	Oct. 31-Nov. 1	122.5	1200	13.5	118	74	90	1148	1562	900	9.1	30	38	38	210	656	31.2
	Nov. 1-2	122.8	1200	13.5	118	74	90	1148	1562	1360	10.6	96	35	62	412	837	25.0
III	2-3	122.5	0	0	0	0	0	0	0	1900	8.0	52	22	44	108	566	20.2
	3-4	122.1	0	0	0	0	0	0	0	2380	8.2	54	24	48	135	761	30.4
	4-5	121.1	0	0	0	0	0	0	0	2645	10.9	73	40	47	220	1115	50.8
	5-6	120.4	0	0	0	0	0	0	0	3000	9.8	88	51	33	202	1050	58.1
	6	119.2															

Patient B. S.

Period I. Fasting with salt mixture.

Period II. 1200 calorie diet.

Period III. Fasting with water only.

Period II. During this period, of six days' duration, the patient was given a diet calculated to yield 1200 calories (C 100, P 80, F 53), which contained by analysis 13.5 gm. N, 118 meq. Na, 74 meq. K, 90 meq. Cl, 1148 mgm. Ca and 1562 mgm. P. Ingested water was maintained at 3000 cc. per day (in addition to the preformed water of the diet which amounted to 1495 gm.).

Period III. During this period, of four days' duration, the patient received 3000 cc. water daily. No food or minerals were given.

RESULTS

The analytic data and the daily weight of the patient are recorded in Table I. Balances during the last three periods are depicted in Table II and Figure 1. In the foreperiod there was a nitrogen deficit of approximately 3 gm. During the first period of starvation, there was a negative nitrogen balance of 45 gm.; and, despite the administration of the mineral salts, there was a negative balance of Na, amounting to 220 meq., of K, 150 meq. and of Cl, 139 meq.

During the subsequent six-day period with a

diet of 1200 calories, there was a positive balance of all these constituents, that for nitrogen amounting to 16 gm., Na 482 meq., K 141 meq. and Cl 239 meq.

In the second fasting period of four days, in which only water was allowed, there was a negative nitrogen balance of 39 gm., of Na, 275 meq., of K, 165 meq. and of Cl, 176 meq. During the first depletion period (salt administration), weight loss was 4 kilos, in the second depletion period (water only) weight loss amounted to 3.3 kilos. During both depletion periods, the patient was hungry, but otherwise complained of no symptoms. He was unable to ascertain that the drinking of the salt mixture, though somewhat disagreeable to take, influenced in any way his general well-being, either for better or worse, as compared with the starvation period without the salts. There were two stools in the period of salt administration, somewhat more liquid than usual for him; whereas stool was obtained only by tap water enema at the end of the starvation period when no salts were ingested. Fecal content of Na, Cl and K indicated that these substances were absorbed. However, the fecal Na was higher in the starvation period with salt administration than we have

estimated to be present in the 1200 calorie diet. Analysis of the diet was not, of course, known until later. The patient ingested considerably more calcium per day on the salt mixture than on the diet.

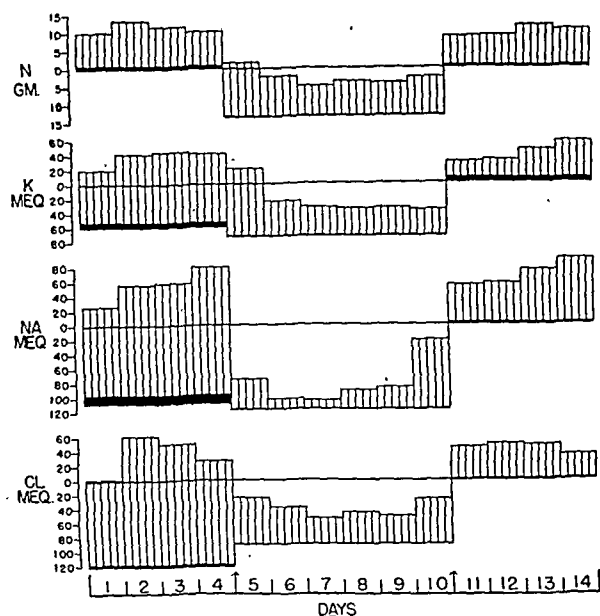


FIG. 1. (PATIENT B. S.) BALANCE DATA FOR N, K, NA AND CL FOR THE THREE PERIODS OF METABOLIC STUDY ARE DEPICTED

The lowest line signifies intake, the difference between upper line and lowest line signifies the outgo. Thus if the uppermost line is above the zero line, this signifies that the overall balance of the substance was negative; if below the zero line, the patient was in positive balance. Heavy black represents the content of the constituent contained in the stool.

Days 1 through 4, the patient was starved but given the salt mixture; days 5 through 10, the patient was fed a 1200 calorie diet; days 11 through 14, the patient was starved and given water only.

seen in other than diarrheal states. The content of potassium in the stool differed little in the two starvation periods.

Case II

J. M. (J. H. H. No. 427946), 28, single, draftsman, sought advice because of obesity. His past history yielded only an attack of inflammatory rheumatism at age 18. He had been overweight as long as he could remember; in the preceding 10 years there had been a gain from 240 to 305 lbs. Physical examination was not remarkable except for extreme obesity, which was general in its distribution. Height was 177.5 cm., weight 137.7 kilos. Examinations of the urine, stool and blood morphology were normal. Basal metabolic rate: plus 2. An electrocardiogram was normal. X-rays of the skull were normal save for a small osteoma on the frontal sinus septum. The blood pressure was 120/80 and was uninfluenced by the dietary procedures carried out. Psychiatric consultation confirmed the opinion of the medical staff that the obesity was related to insecurity with resulting phagomania.

The regimens followed on this patient were in general the same as those of Case I, except that in the first period of fasting he was given water only; during the second period water and the salts were given. The salt mixture given to this patient was supposed to be the same as that given to B. S. but on analysis contained much less calcium.³

³ The relatively smaller amount of calcium in the salt mixture given to this patient was not intended. It occurred because, in making up the mixture, dibasic calcium phosphate was inadvertently substituted for the tribasic salt.

TABLE II
Balance data

Per.	Date	Stool						Balance				Serum							Sugar	Uric acid	Htct.
		N	Na	K	Cl	Ca	P	N	Na	K	Cl	Na	K	Cl	HCO ₃	Ca	P	NPN			
		gm.	meq.	meq.	meq.	mgm.	mgm.	gm.	meq.	meq.	meq.	meq./l.	meq./l.	meq./l.	meq./l.	mgm. %	mgm. %	mgm. %			
I	Oct. 23-24	.8	12	8	4	156	164	-10.0	-26	-20	-1	141	3.9	106	27.6	10.0	4.5	32	114	5.3	51
	24-25	.8	12	8	4	156	164	-13.4	-56	-42	-62										
	25-26	.8	12	8	4	156	164	-11.7	-58	-45	-50										
	26-27	.8	12	8	4	156	164	-10.3	-80	-43	-26										
II	27-28	.2	1	2	1	247	174	-1.6	75	-22	25	143	4.5	104	22.8	12.2	5.4	34	68		51
	28-29	.2	1	2	1	247	174	2.2	103	24	88										
	29-30	.2	1	2	1	247	174	5.0	105	33	53										
	30-31	.2	1	2	1	247	174	3.8	91	35	45										
	Oct. 31-Nov. 1	.2	1	2	1	247	174	4.2	87	34	51										
	Nov. 1-2	.2	1	2	1	247	174	2.7	21	37	27										
III	2-3	.5	2	7	1	591	376	-8.5	-54	-29	-45	140	4.1	109	27.6	11.1	3.4	29	124	5.1	48
	3-4	.5	2	7	1	591	376	-8.7	-56	-31	-49										
	4-5	.5	2	7	1	591	376	-11.4	-75	-47	-48										
	5-6	.5	2	7	1	591	376	-10.3	-90	-58	-34										
6												137	4.3	105	21.6	9.8	3.7	32	66	11.1	48

Patient B. S.

Period I. Fasting with salt mixture.

Period II. 1200 calorie diet.

Period III. Fasting with water only.

RESULTS

The analytical data and daily weight are recorded in Table III. Balance data are given in Table IV and graphically recorded on Figure 2. During the four-day foreperiod, with a diet of 1200 calories, there was a nitrogen deficit of approximately 14 gm. In Period I, when only water was allowed for four days, nitrogen loss amounted to 60.6 gm., potassium 250 meq., sodium 205.0 meq. and chloride 212 meq. Acetonuria (Rothera test) was found to be 4 plus at the end of this period.

During Period II, six days when 1200 calories daily were given, there was a further nitrogen loss of 2.4 gm.; but potassium, sodium and chloride were retained in amounts of 142, 420 and 258 meq., respectively. Traces of acetone continued to be found in the urine during this entire period.

In Period III, when the patient was fasted and salts were administered with the water, there occurred a negative balance of all constituents; nitrogen 35.5 gm., K 21.0 meq., Na 176.0 meq., and Cl 118.0 meq. During both periods of starvation the only symptoms complained of by the patient were hunger, weakness and brief morning headache, none of which were more than mildly an-

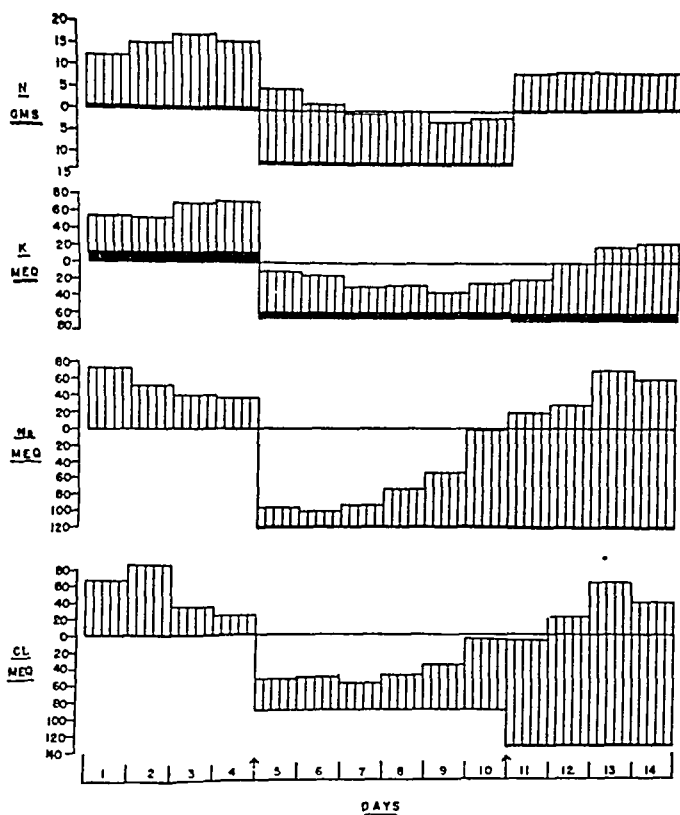


FIG. 2. (PATIENT J. M.) BALANCE DATA ARE CHARTED AS IN FIGURE 1

Days 1 through 4, the patient was starved and given water only; days 5 through 10, the patient was fed a 1200 calorie diet; days 11 through 14, the patient was starved and given the salt mixture.

TABLE III
Analytic data

Per.	Date	Wt.	Cal.	Intake						Urine vol.	Urine					
				N	Na	K	Cl	Ca	P		N	Na	K	Cl	Ca	P
		<i>kilos</i>		<i>gm.</i>	<i>meq.</i>	<i>meq.</i>	<i>meq.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>cc.</i>	<i>gm.</i>	<i>meq.</i>	<i>meq.</i>	<i>meq.</i>	<i>mgm.</i>	<i>mgm.</i>
I	July 14-15	132.5	0	0	0	0	0	0	0	680	11.3	73	44	67	98	418
	15-16	130.9	0	0	0	0	0	0	0	1180	14.4	52	42	84	136	688
	16-17	130.5	0	0	0	0	0	0	0	2180	16.5	39	59	34	220	1230
	17-18	129.5	0	0	0	0	0	0	0	2020	14.8	37	61	23	272	1180
II	18-19	128.2	1200	12.64	119	67	91	1128	1586	2020	17.2	22	48	37	232	1120
	19-20	128.0	1200	12.64	119	67	91	1128	1586	1680	13.8	19	45	38	218	895
	20-21	127.7	1200	12.64	119	67	91	1128	1586	1840	11.6	25	31	31	137	806
	21-22	128.6	1200	12.64	119	67	91	1128	1586	1960	12.2	40	32	42	183	760
	22-23	129.3	1200	12.64	119	67	91	1128	1586	1620	9.8	64	25	54	183	578
	23-24	129.4	1200	12.64	119	67	91	1128	1586	1880	10.6	118	37	86	162	860
III	24-25	129.2	0	0	121	70	135	2300	1630	880	8.2	137	43	127	106	543
	25-26	128.9	0	0	121	70	135	2300	1630	1840	8.7	148	63	156	131	737
	26-27	127.9	0	0	121	70	135	2300	1630	2260	8.5	189	81	197	137	904
	27-28	127.0	0	0	121	70	135	2300	1630	1800	8.5	178	86	174	294	960
	28	126.0														

Patient J. M.

Period I. Fasting with water only.

Period II. 1200 calorie diet.

Period III. Fasting with salt mixture.

TABLE IV
Balance data

Per.	Date	Stool						Balance				Serum							Uric acid	Htct.	T.P.	A/G
		N	Na	K	Cl	Ca	P	N	Na	K	Cl	Na	K	Cl	HCO ₃	Ca	P	NPN				
I	July 14-15	.9	.5	11	.6	178	126	-12.2	-74	-55	-68	140	3.7	104.4	27.9	10.7	3.7	32	8.0	47	7.7	5.4/2.3
	15-16	.9	.5	11	.6	178	126	-15.3	-53	-53	-85											
	16-17	.9	.5	11	.6	178	126	-17.4	-40	-70	-35											
	17-18	.9	.5	11	.6	178	126	-15.7	-38	-72	-24											
II	18-19	.5	1.0	7	.2	139	79	- 5.0	+96	+12	+54	136.7	4.0	95.4	23.3	11.1	4.0	31	10.7	47	8.0	5.5/2.5
	19-20	.5	1.0	7	.2	139	79	- 1.7	+99	+15	+53											
	20-21	.5	1.0	7	.2	139	79	+ 0.6	+93	+29	+60											
	21-22	.5	1.0	7	.2	139	79	- 0.1	+78	+28	+49								8.0			
	22-23	.5	1.0	7	.2	139	79	+ 2.3	+54	+35	+37								6.7			
	23-24	.5	1.0	7	.2	139	79	+ 1.5	0	+23	+ 5								7.6			
III	24-25	.4	2.0	7	1.0	308	136	- 8.6	-18	+20	+ 7	137.5	4.0	101.0	27.9	11.1	3.7	25	6.1	46	7.1	4.9/2.2
	25-26	.4	2.0	7	1.0	308	136	- 9.1	-29	0	-22											
	26-27	.4	2.0	7	1.0	308	136	- 8.9	-70	-18	-63											
	27-28	.4	2.0	7	1.0	308	136	- 8.9	-59	-23	-40											
	28											135.5	3.8	99.0	26.6	11.1	3.8	38	10.0	46	7.6	5.3/2.3

Patient J. M.

Period I. Fasting with water only.

Period II. 1200 calorie diet.

Period III. Fasting with salt mixture.

noying. He found no discomfort from taking the salt mixture, and believed it allayed his hunger somewhat. Acetonuria increased, and at the end of the period was found to be 3 plus. Five stools were passed during this period, in contrast to one stool during Period I.

DISCUSSION

Administration of the salt mixture in both cases was accompanied by an increase in the number of stools passed. B. S. had two stools in the four days of starvation with the salts, both of which were somewhat more liquid than usual for him; whereas no stools were passed in his other period of starvation and his colon was evacuated by tap water enema at the end of the period. J. M. passed five stools with the salt mixture, but only one during starvation without the salts. In both cases the amount of K, Na and Cl recovered from the stools was but little affected by the salt mixture, indicating almost complete absorption of these elements. Nor was the stool content of nitrogen altered.

Both patients lost weight, nitrogen, and presumably some potassium, sodium and chloride during their foreperiods. B. S. lost 3.5 kilos in weight, J. M. lost 5 kilos; so that both began their total starvation periods in approximately equal status. In both cases also, the six-day feeding

period failed to restore either the weight or the previously lost nitrogen, so that both patients began their second total starvation periods with less weight and less total nitrogen than was present at the beginning of the first starvation periods. It has been noted that undernourished and chronically debilitated persons can be maintained in nitrogen equilibrium on lower intake of both calories and nitrogen than normal, healthy, vigorous persons (4, 5). As a corollary, an individual would be expected to lose less nitrogen when subjected to a fast which began when he had already lost some nitrogen, than when subjected to a fast begun in full state of nutrition. Benedict's fasting man lost less nitrogen per day as the fasting progressed (1). Our patients lost less nitrogen in the second fasting period than in the first (patient B. S. lost 6 gm. less and patient J. M. lost 25 gm. less), though in both cases the weight losses in the two fasting periods were the same. Since the salt mixture was given to B. S. in his initial period of fasting and to J. M. in his second period of fasting, it may be concluded that the administration of the salt mixture did not exert any appreciable sparing effect on the nitrogen balance during starvation, nor did it appreciably alter the amount of weight lost.

Comparison of the potassium balance in the starvation periods, with and without ingestion of the salt mixtures, reveals that the results in the two

patients were not identical. B. S., to whom the salt mixture was given in his first starvation period and water only in the second starvation period, sustained losses of potassium that were almost identical—150 meq. and 165 meq., respectively. This patient began each fasting period with approximately the same amount of potassium in his body, since, in the six-day interval when he was fed, there had been replacement of all but 9 meq. of the potassium lost in the initial fast. The greater loss of potassium was exhibited in the period during which less nitrogen was lost; so that one might conclude that the salt mixture had effected a small saving of potassium to the organism.

In the case of J. M., the salt mixture was given during the second period of starvation, and seemingly effected a marked sparing action on the amount of potassium lost. During Period I there was a deficit of 250 meq. K; during Period III the loss was only 21 meq. This patient had not retained nitrogen during his six-day interval when fed, and had only partially repleted the potassium lost in the initial fast. Thus, in this patient, administration of the salt mixture was accompanied by a large saving of potassium during his four-day fasting period. Whether or not this sparing of body stores of potassium was beneficial to the patient cannot be stated; there was no detectable difference in his symptoms during the two fasting periods, and weight loss was practically identical. The full repletion of potassium during the feeding interval in B. S., far greater than the comparative repletion of nitrogen; and the repletion of 142 meq. of the previously lost 250 meq. of potassium by J. M. during the feeding interval, even in the face of negative nitrogen balance, furnish corroborative evidence of our previous observation that, after brief periods of starvation, potassium is repleted more rapidly than nitrogen (3).

Both patients lost large quantities of sodium and chloride in both starvation periods; the administration of the salt mixture appeared to influence the magnitude of these losses but little. In both patients, during the intervening period when they were on a 1200 calorie diet with average Na intake, there was retention of this element amounting to twice the losses sustained in the first starvation period. This over-repletion

of these extracellular elements is in line with previous observations that, during total intravenous feeding before and after surgical operations, the amounts of sodium and chloride retained varies widely, depending on the general nutritional state and the status of hydration at the time (6). Both the patients in this study thus began their second period of starvation with more sodium and chloride in their bodies than was present at the beginning of the first starvation period. Both patients lost a little less of these elements when the salt mixture was given, and since one of the patients received the salts in his first fast while the other received them in the second, one may conclude that the administration of salts effected a slight reduction in the overall losses of sodium and chloride. However, when one compares the balance of sodium and chloride *on the first day of starvation* with and without the salt mixture, there is a striking difference, the losses in both instances having been almost negated by the salt administration. Except for Period I in which J.M. lost 205 meq. sodium and 212 meq. chloride (ratio 1 : 1), the movement of these two electrolytes was in general parallel, with approximate ratio of 3 Na to 2 Cl, as in extracellular fluids. As previously mentioned, there was no appreciable difference in the weight losses sustained by either patient as the result of the administration of the salt mixture.

The overall movements of calcium and phosphorus are exceedingly difficult to interpret in balance studies of brief duration. When dietary calcium and phosphorus are abruptly changed from high intake to low and vice versa, there has been observed a highly variable "lag period" before the stool analyses reflect the altered diet (7). This is presumably due to the fact that all the calcium and phosphorus which will eventually reach the rectum may not traverse the gut at a uniform speed, certainly not coincident with the movement of carmine which is usually used to mark beginning and ending of metabolic periods. For these reasons no attempt will be made here to interpret balance data on these two substances. However, the urinary content of phosphorus during the starvation periods seemed to be affected little or none by the administration of the salt mixture to either patient. The urine of B. S. contained more phosphorus when he ingested the mixture than

when he did not; on the other hand, the reverse was true in the case of J. M. It seems more likely that little or none of the phosphorus of the mixture was absorbed (since there was considerable excess of calcium), and that the urinary phosphorus was a reflection of its movement with nitrogen; for the urinary phosphorus was greater in both patients in their first starvation period when nitrogen losses were greater.

There was no appreciable effect of the salt mixture on the urinary excretion of calcium in the case of J. M., but B. S., who received the greater amount of calcium in the salt mixture, exhibited a heightened calciuria of 150 mgm. per day when given the salt mixture, which was interpreted as an indication that some of the calcium had been absorbed.

Concentration in the serum of the various ions under consideration followed, in the main, the expected pattern. There was no appreciable change in non-protein nitrogen during the starvation periods. Serum bicarbonate fell slightly in all four fasting periods. Concentration of potassium rose very slightly except in the second starvation period of J. M. (in which he received the salt mixture). The concentrations of sodium and chloride fell slightly in all four fasting periods, seemingly uninfluenced by the administration of the salt mixture. The serum concentration of calcium remained stable in J. M., but rose 2 mgm. per 100 cc. in B. S. during starvation when he was given the salt mixture. This was reflected in a considerable increase in calciuria coincidently. The rise in serum calcium in this period is all the more striking when compared with its behavior during B. S.'s second fast, during which there was a fall in serum calcium. The conclusion seems inescapable that there was appreciable absorption of calcium from the salt mixture.

Fasting blood sugar concentration fell 40 mgm. per 100 cc. in each of the starvation periods of B. S., the mild diabetic. Uric acid (8) rose in the serum of both patients to high levels during the fasting periods, as previously noted in undernutrition regimes in normal persons (3).

SUMMARY

Two obese male patients were subjected to two periods each of four days' starvation. During one

fast each patient received only water; in the other a salt mixture, containing approximately the amounts of sodium, chloride, potassium and phosphorus of a 1200 calorie diet, was given in addition to the water. Between the two fasting periods both patients were fed for six days a diet containing 1200 calories.

Administration of the salt mixture resulted in no lessening of the nitrogen lost by fasting and effected no alteration in the amount of weight lost. In one patient there appeared to be considerable saving of potassium from the salt mixture, in the other patient none.

On the first day of the fasts, large savings of sodium and chloride were manifest from the salt mixtures, but thereafter excretion of these elements was rapid; and the total amounts of sodium and chloride lost over the four days were the same in the fasting periods with and without the salt mixtures.

The sodium, chloride and potassium of the mixtures were almost completely absorbed, as judged from the quantities of these elements in the stools and by the increased amounts appearing in the urine. There was no appreciable rise in phosphaturia when the salt mixture was given, as compared with fasting alone. In one of the patients there was heightened calciuria and hypercalcemia as a result of the salt mixture.

The concentrations in the serum of sodium, chloride and potassium were uninfluenced by the salt administration.

Stools were more frequent during fasting when the salt mixture was given, but only minimal amounts of the administered salts were recovered in the stools.

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THE RELATION OF ALBUMIN TO PRECIPITABLE IODINE OF SERUM¹

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By correlation with clinical manifestations of thyroid disease, by comparison with basal metabolism, and serum lipids, and by studies of the effects of removal of the thyroid and the administration of thyroid substance and thyroxine, it has been established that the precipitable iodine of the blood serum is, under usual circumstances, a most accurate measure of the activity of the thyroid gland (1 to 6). There is, indeed, reason to believe that the precipitable iodine may be largely, if not chiefly, composed of thyroid hormone. Thyroxine, added to normal serum, attaches itself so firmly to the proteins that it cannot be detached by washing (2). There is some evidence that the fraction of protein with which the iodine is combined is albumin (1). If this is established, this fraction may be regarded as the normal vehicle for the thyroid hormone.

In the serum of certain patients with extreme hypoalbuminemia concentrations of precipitable iodine as low as those found in myxedema have been encountered. The present paper deals with an examination of the incidence and significance of this serum iodine deficiency.

METHODS

From venous blood, drawn from patients in the post-absorptive state, serum was separated with anaerobic precautions. Proteins and fractions were measured by the method of Howe (7) up to January 27, 1947, after which Milne's (8) modification of the procedure, according to the principles of Majoor (9) was employed. This modification yields values which are lower for albumin and higher for globulin than those given by the original Howe technique, agreeing closely with the results of electrophoretic analyses. Serum lipids were measured by the methods of Man, Gildea, Peters and Bogdanovitch (10 to 14). Serum precipitable iodine was measured by the methods of Man and Riggs (2).

¹ Aided by a grant from the U. S. Public Health Service.

RESULTS

The observations about which the discussion will center are presented in Table I. The coincidence of low serum iodine with extreme hypoalbuminemia was first noted in patient 576, suffering from chronic pyelonephritis with profuse albuminuria as well as biliary cirrhosis. This case will be discussed at greater length below. It was next encountered in two patients with the nephrotic syndrome, B74160 and B95522. Subsequently it was demonstrated in a series of patients with miscellaneous conditions in which hypoalbuminemia appeared to be the only common factor. In a certain number of these patients the effects of thyroid therapy and of injections of salt-poor human albumin upon serum iodine were studied.

DISCUSSION

A review of all patients who have had simultaneous determinations of iodine and albumin reveals no direct correlation between these two variables. This is evident from Figure 1. There is, to be sure, a large aggregation of points in the lower left-hand corner in which both iodine and albumin are reduced together, but these represent multiple observations on the group of subjects now under consideration. Patients with myxedema and minimal quantities of precipitable iodine in their sera may have normal or high concentrations of serum albumin. The correlation between the precipitable iodine of serum and the state of thyroid activity in patients with hyper- and hypothyroidism has been demonstrated at length in previous publications (2 to 6). Although serum proteins were not measured in the majority of these patients, there is no evidence, when it was, that the precipitable iodine was modified by the concentration of serum albumin.

Epstein (15, 16) in 1917 suggested that patients with the nephrotic syndrome had thyroid defi-

TABLE I
Serum precipitable iodine in patients with hypoalbuminemia

Sex	Age	Date	Pptble. I	Pro- tein	Albu- min	Glob- ulin	Cholesterol		Fatty acid	Lipid P	Basal metab.	Diagnosis and remarks
							Total	Free				
			γ per cent	per cent	per cent	per cent	mg. per cent	mg. per cent	mEq. per liter	mg. per cent	per cent	
B74160 F	17	1946										<i>Nephrotic syndrome</i> After thyroid gr. 3 daily for 11 days After thyroid gr. 3 daily for 23 days After thyroid gr. 3 daily for 31 days After thyroid gr. 4 daily for 14 days After thyroid gr. 4 daily for 40 days Thyroid gr. 4 daily stopped after 2/19 Just before albumin injections After 125 gm. albumin After 275 gm. albumin After 525 gm. albumin After 475 gm. albumin
		11/14	1.5	3.53	1.20	2.33	—	—	—	—	—	
		12/4	0.7	3.79	1.21	2.58	—	—	—	—	—	
		12/16	—	3.93	1.17	2.76	—	—	—	—	—	
		12/24	1.4	—	—	—	631	183	60.5	24.1	—	
		1947										
		1/15	—	3.85	1.20	2.65	—	—	—	—	—	
		2/10	—	3.85	0.75	3.10	—	—	—	—	—	
		2/24	1.0	—	—	—	627	180	60.0	22.1	—	
		3/3	—	4.07	0.62	3.45	—	—	—	—	—	
		3/7	—	5.02	1.76	3.26	—	—	—	—	—	
		3/10	2.0	5.47	2.38	3.09	596	—	51.4	19.9	—	
		3/15	3.8	5.23	2.13	3.10	471	—	39.7	19.6	—	
		3/20	—	4.27	0.80	3.47	—	—	—	—	—	
		3/27	3.2	4.08	0.84	3.24	583	168	44.8	21.5	—	
		4/3	2.6	4.66	0.98	3.68	388	—	25.0	—	—	
		4/14	1.7	3.98	0.84	3.14	553	147	41.9	23.2	—	
		7/3	2.3	4.86	0.85	4.01	—	—	—	—	—	
		7/14	2.4	5.27	2.36	2.91	445	—	28.5	—	—	
B95522 M	32	1947										<i>Nephrotic syndrome</i> After 325 gm. albumin After 675 gm. albumin After thyroid gr. 2 daily for 14 days After thyroid gr. 4 daily for 14 days On thyroid gr. 2 daily After 500 gm. albumin
		4/8	2.8	3.27	1.51	1.76	468	147	26.5	20.5	—	
		4/20	—	4.75	2.30	2.45	—	—	—	—	—	
		4/27	2.9	4.91	3.39	1.52	267	97	21.3	17.2	—	
		5/5	3.2	4.13	2.22	1.91	255	88	19.7	16.2	—	
		5/23	2.8	—	—	—	323	95	23.8	18.0	—	
		6/11	1.9	3.79	1.52	2.27	260	95	21.4	19.6	—	
		7/11	2.1	3.93	1.11	2.82	—	—	—	—	—	
		7/29	—	3.05	0.76	2.29	—	—	—	—	—	
		7/30	0.7	—	—	—	—	—	42.7	25.9	—	
		8/15	0.3	3.63	1.53	2.10	594	153	31.0	—	—	
B72419 M	28	1947										<i>Intercapillary glomerular sclerosis. Diabetes</i> After 300 gm. albumin
		6/12	—	3.96	1.16	2.80	—	—	—	—	—	
		6/16	4.4	—	—	—	367	99	20.7	13.8	—	
		6/23	—	6.02	3.01	3.01	—	—	—	—	—	
		6/24	1.8	—	—	—	316	84	24.9	—	—	
576 F	64	1945										<i>Chronic pyelonephritis. Biliary cirrhosis. Myxedema?</i> After thyroid gr. 1 daily for 11 days After thyroid gr. 3 daily for 18 days After thyroid gr. 4 daily for 2½ mos. Still taking thyroid gr. 4 daily Still taking thyroid gr. 4 daily On thyroid gr. 3 daily since 5/8 Still taking thyroid gr. 3 daily
		4/16	—	3.24	1.15	2.09	—	—	—	—	—	
		4/20	1.3	—	—	—	562	—	32.7	—	—	
		4/26	—	3.10	1.10	2.00	—	—	—	—	-12	
		5/8	—	5.91	1.00	4.91	—	—	—	—	—	
		5/9	1.3	—	—	—	589	206	65.0	21.7	—	
		6/6	3.4	—	—	—	552	176	33.4	18.4	—	
		9/5	—	3.34	1.38	1.96	365	130	23.4	12.5	—	
		10/26	5.0	4.27	1.85	2.42	352	120	28.3	15.1	—	
		1946										
		5/1	5.1	3.63	1.50	2.13	201	73	12.8	8.8	—	
		5/21	5.0	3.90	1.58	2.32	—	—	—	—	—	
		6/3	2.8	4.11	2.21	1.90	—	—	—	—	—	
B44829 F	75	1947										<i>Diabetes. Cirrhosis of liver, probably biliary</i> After 275 gm. albumin After 275 gm. albumin
		7/31	—	—	—	—	170	65	15.6	9.5	—	
		8/15	—	5.73	1.93	3.80	—	—	—	—	—	
		8/21	—	6.17	3.44	2.73	—	—	—	—	—	
		8/28	—	6.52	3.98	2.54	61	31	7.3	5.2	—	
B96023 M	59	1947 4/17	4.2	7.17	1.33	5.84	163	81	13.2	11.2	—	<i>Cirrhosis of the liver</i>

TABLE I—Continued

Sex	Age	Date	Pptble. I	Pro- tein	Albu- min	Glob- ulin	Cholesterol		Fatty acid	Lipid P	Basal metab.	Diagnosis and remarks
							Total	Free				
			γ per cent	per cent	per cent	per cent	mg. per cent	mg. per cent	mEq. per liter	mg. per cent	per cent	
B78803 M	62	1946 4/10 5/16	— —	5.89 6.10	2.66 3.20	3.23 2.90	129 92	47 41	9.3 9.4	8.2 7.7	— —	<i>Cirrhosis of the liver</i> After 462 gm. albumin
B82147 M	33	1947 6/16 7/7	3.0 1.9	4.20 3.97	1.61 2.66	2.59 1.31	141 79	43 25	11.0 7.6	— —	— —	<i>Calcified pericardium</i> After 800 gm. albumin
B97275 F	63	1947 6/4 6/17	5.2 3.8	3.02 4.23	1.37 2.66	1.60 1.57	115 —	42 —	9.2 —	— —	— —	<i>Vomiting and diarrhea, unexplained. Nutri- tional edema</i> After 475 gm. albumin
12377 F	44	1947 3/17	3.7	4.56	0.93	3.63	58	20	5.2	6.1	—	<i>Exfoliative dermatitis</i>
A76963 M	45	1947 9/22 9/24 9/29 10/2 10/6 10/8 10/14 10/21	— 3.0 — — 7.0* — — — 3.5	5.33 — 5.76 5.69 — 5.79 6.23 —	1.26 — 1.21 1.23 — 1.50 2.97 —	4.07 — 4.55 4.46 — 4.29 3.26 —	— — — — 241 — — 268	— — — — 69 — — 78	— — — — 11.3 — — 12.8	— — — — 10.7 — — 10.9	— — — — — — —13 —	<i>Thrombophlebitis. Nephrotic syndrome</i> After 175 gm. albumin After 275 gm. albumin After 525 gm. albumin 4 days after 575 gm. albumin
71570 M	59	1947 7/22 9/9	2.6 2.0	4.95 5.19	2.10 1.79	2.85 3.40	108 —	25 —	6.4 —	6.3 —	— —	<i>Craniopharyngeoma. Pituitary deficiency?</i> After thyroid gr. 2 daily for 14 days
A86708		1942 6/3	5.6†	6.82	—	—	—	—	—	—	-22	<i>Anorexia nervosa</i>
P3027		1943 1/8 2/11 10/23	3.7† 3.6† 4.8†	4.96 5.68 6.07	3.83 3.72 —	1.13 1.96 —	199 176 —	64 — —	10.9 9.6 —	9.1 — —	-33 -25 -25	<i>Anorexia nervosa</i>
B10436		1944 1/28	5.6	5.59	4.32	1.27	129	41	11.5	7.5	—	<i>Anorexia nervosa</i>
B71694		1945 8/23	4.7	6.73	4.09	2.64	—	—	—	—	-28	<i>Anorexia nervosa</i>
B6589		1945 8/10 8/21	— 5.4	6.16 —	4.42 —	1.74 —	— —	— —	— —	— —	— —	<i>Anorexia nervosa</i>
B77426		1946 4/4	3.1	7.00	5.14	1.86	—	—	—	—	-24	<i>Anorexia nervosa?</i>
B74923 M	29	1946 2/7 2/12 3/27 5/3 6/1 1947 8/6	3.3 — — 3.6 6.7 — 1.7	— — — 6.72 — — —	— — — 4.49 — — —	— — — 2.23 — — —	— — — 206 — — —	— — — 66 — — —	— — — 29.8 — — —	— — — 12.0 — — —	— -31 — — — -22 -29	<i>Hypopituitarism</i> On thyroid gr. 1 daily On thyroid gr. 1 daily On thyroid gr. 1 daily

TABLE I—Continued

Sex	Age	Date	Pptble. I	Pro- tein	Albu- min	Glob- ulin	Cholesterol		Fatty acid	Lipid P	Basal metab.	Diagnosis and remarks
							Total	Free				
			γ per cent	per cent	per cent	per cent	mg. per cent	mg. per cent	mEq. per liter	mg. per cent	per cent	
B76181 M	16	1946										<i>Hypopituitarism</i>
		1/21	—	6.70	—	—	—	—	—	—	—	
		1/26	3.4	—	—	—	—	—	—	—	—	
		3/21	2.9	—	—	—	229	—	10.9	—	—	
		1947										On thyroid gr. 3 daily On thyroid gr. 4 daily On thyroid gr. 4 daily
		1/13	3.2	—	—	—	146	—	7.9	—	—19	
		8/4	3.9	—	—	—	—	—	—	—	—	
		8/21	—	6.39	3.62	2.77	—	—	—	—	—	
		10/31	1.3	—	—	—	—	—	—	—	—	
B69216 M	44	1945										<i>Hypopituitarism</i>
		5/25	1.7	—	—	—	—	—	—	—	—29	
		5/31	—	—	—	—	247	—	12.3	9.3	—	On thyroid gr. 2 daily On thyroid gr. 2 daily
		6/18	4.7	—	—	—	—	—	—	—	—33	
		11/22	2.2	—	—	—	—	—	—	—	—	
B98193 M	47	1947										<i>Hypopituitarism</i>
		9/19	2.9	—	—	—	—	—	—	—	—	
		10/4	—	6.28	3.77	2.51	281	80	16.4	11.7	—	
		10/14	5.1	—	—	—	—	—	—	—	—	

* This sporadic high figure is quite inexplicable.

† Figures for total serum iodine instead of precipitable iodine.

ciency and were benefitted by administration of thyroid substance. He based this opinion upon the low basal metabolism and high serum cholesterol that commonly occur in this condition. These chemical disorders are not, however, associated with the clinical symptoms or signs of hypothyroidism. Furthermore, it has been pointed out that the lipemia of the nephrotic syndrome differs from that of myxedema. In the former, neutral fat is increased, as well as cholesterol and phospholipids, while in myxedema the increment of fatty acid appears to be derived entirely from cholesterol esters and phospholipids (17). These features are well illustrated by B74160 of this series (B95522 is not so characteristic because his nephrosis was complicated by thrombophlebitis and liver disease). The patient B74160 was a vivacious girl of 18, mentally alert and physically rather hyperactive, with a variable, but persistent, tachycardia. Her skin and hair were normal in texture, her vasomotor reactions somewhat excessive. Presumably her basal metabolism was low. Her serum lipids were greatly elevated, with more than the usual proportion of fatty acids. The administration of dried thyroid (U.S.P.), in doses as great as 4 grains per day over long periods, had no appreciable effect upon the lipids

of her serum nor did it cause the serum precipitable iodine to rise appreciably. It was impossible to determine whether it influenced her heart rate because as was remarked above, she had a variable, but persistent, tachycardia at all times. The tachycardia while she was taking 4 grains of thyroid daily was no greater than it was at some other times when she was receiving no thyroid. The thyroid had no detectable effect upon her weight or edema. This in itself distinguishes her from the true hypothyroid patient who reacts sharply and quantitatively to smaller doses of thyroid (3, 5). Patient B95522 reacted, or failed to react, in a similar manner to an equally large dose of thyroid. His serum lipids were not, however, as distinctly elevated as those of B74160. The ratio of free to total cholesterol in his serum was slightly above normal, and the proportion of fatty acid was not excessive. These modifications of the usual nephrotic pattern were probably referable to the hepatic disorder.

It seemed possible that the low serum iodine represented not a diminution in the production of thyroid hormone or the amount available to the tissues, but merely a deficiency of the vehicle, serum albumin, by which it is carried in the blood stream. The reaction to the injection of salt-poor

human serum albumin was, therefore, examined.² This albumin itself contained minimal amounts of iodine. Analysis yielded 3.0 to 4.6 γ per 100 cc., containing 25 gm. of albumin. If the precipitable iodine of serum is held entirely by the albumin fraction of the proteins, 25 gm. of normal serum albumin should contain from 24 to 48 γ . The first time that albumin was administered to B74160 her serum iodine seemed to rise slightly, from 1.0 to 3.8 γ per cent; but on subsequent occasions in this subject and on almost all occasions in other subjects no such response was observed. In fact, in most instances the serum iodine tended to fall.

Patients with nephrosis do not constitute the most suitable subjects for the examination of the

² The serum albumin used in the research described herein was furnished by the Administrator of the National Blood Program, American Red Cross, on recommendation of the Subcommittee on Blood and Blood Derivatives of the Red Cross Medical Advisory Committee.

effects of injections of serum albumin because the material is so rapidly excreted in the urine that its effect on the concentration of albumin in the serum is quite evanescent. By daily injections of the albumin it was, however, possible to maintain the serum albumin of B74160 for a week about 1.5 per cent above its initial value. This should have been long enough to allow some kind of new equilibrium to be established.

In the nephrotic syndrome excretion in the urine of iodine combined with protein might contribute to the serum iodine deficiency. Measurement of the iodine attached to urinary protein proved to be a difficult technical problem because it required the treatment of such large volumes of material. A 24-hour sample of urine from B74160 contained 11.70 gm. of protein which, in turn, held 16.05 γ of iodine, or 1.37 γ of iodine per gram of protein. The serum, by the Howe technique, contained 3.9 and 1.2 per cent of total protein and albumin, respectively, and 1.4 γ per cent of pre-

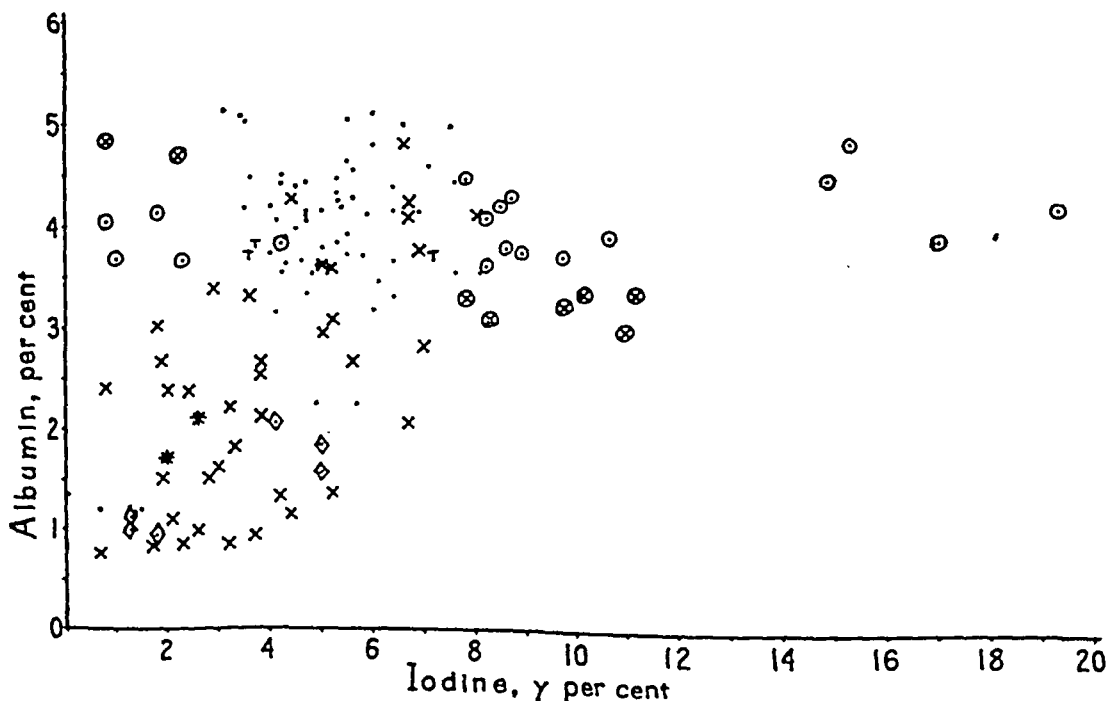


FIG. 1. THE RELATION OF PRECIPITABLE IODINE TO ALBUMIN OF SERUM

- Albumin by Howe method.
- ×—Albumin by Milne-Major method.
- T—Albumin by Howe method, total serum iodine.
- Open circles about dots and crosses represent patients with myxedema and hyperthyroidism.
- Diamonds mark determinations upon patient No. 576, discussed in the text.
- Stars mark the determinations on patient No. 71570, also discussed in the text.

capitable iodine. The serum therefore contained $1.4/3.9 = 0.36 \gamma$ of iodine per gram of protein or 1.17γ of iodine per gram of albumin. The urine of 576 on one occasion contained 1.7 to 3.0γ of iodine per gram of protein, while the serum contained 0.52γ per gram of total protein or 2.0γ per gram of albumin. Without more precise knowledge of the partition of protein fractions in serum and urine, respectively, the proportions of iodine to protein in the two media cannot be accurately compared. If all the protein in the urine was albumin, which can hardly have been the case, and all the iodine in the serum was bound by albumin, urine albumin and serum albumin held approximately the same amounts of iodine. Compared on the basis of total protein in the two media, more iodine was held by the urine protein than by the serum protein in both instances. This might be anticipated from the known differences between the electrophoretic patterns of serum and urine in the nephrotic syndrome (18). The actual quantities of iodine lost in 24 hours, from 16 to 48γ , are far smaller than the hormonal requirements of normal individuals estimated from the amounts of thyroid substance or thyroxine needed to maintain a patient with myxedema in a euthyroid state. Although losses of iodine with protein in the urine may, in nephrosis, contribute to the depletion of serum precipitable iodine, they cannot be wholly accountable for it. Moreover, they can not account in any degree for the serum iodine deficiency in cirrhosis of the liver and the other conditions in which it is found in association with hypoalbuminemia in the absence of proteinuria.

From Figure 1 precipitable iodine appears to be affected by serum albumin only when the latter is profoundly reduced. The clinical material from which this figure is composed is, however, so variable that it could not be asserted with any assurance that lesser variations of serum albumin have no influence upon serum iodine. It is conceivable that the serum iodine deficiency is connected not only with the degree, but also with the nature, of the albumin deficit. It has been shown that in the hypoalbuminemia of the nephrotic syndrome and of cirrhosis of the liver the two chief fractions of serum albumin do not suffer equal depletion. It might be anticipated that if only one fraction, the vehicle of iodine, was depleted, in-

jection of whole serum albumin would restore the vehicular capacity of the serum. The major proportion of the injected albumin does not, however, remain in the circulation. The salt-poor human albumin employed for these injections contains far less iodine than normal circulating albumin is believed to carry. It has, in fact, been suggested that iodine is carried not by albumin but by α -globulin. In this case injection of albumin could not increase, but should rather tend to diminish its concentration in serum. On the other hand, this would make the deficiency of serum precipitable iodine in hypoalbuminemia the more inexplicable, since α -globulin usually increases when albumin diminishes.

When the serum albumin of the nephrotic subjects rose under the influence of injections of albumin, the lipids fell without any disturbance of the relative proportions of the various fractions. Barker and Kirk (19) in 1930 reported that serum protein and cholesterol varied inversely in dogs subjected to plasmapheresis. This was not confirmed by Leiter (20). That there is no consistent inverse relation between these variables in disease in general has been amply demonstrated. In malnutritional states with hypoalbuminemia the serum lipids are usually reduced (21). This is true also in hepatic cirrhosis and advanced degeneration of the liver (22). Both also fall together after operations (23). In renal hyperlipemia itself there is no consistent inverse correlation between the albumin and lipids of the serum. It is clear from Table I that in all these conditions injections of albumin had a similar effect: the serum lipids fell, whether they were initially high or low. The reductions thus induced cannot be interpreted as evidence of improvement in nephrosis since it occurred equally in all patients, including those with cirrhosis of the liver and hypolipemia, whether there was a concomitant diuresis or not. These reductions must be attributed not to any action that albumin may exert upon the underlying disease, but rather to some more specific action upon the chemical structure of the serum. They cannot be ascribed simply to hemodilution because they are altogether too great in many instances and are not proportional to the decreases of other elements such as globulin. Furthermore, they appear to outlast the other effects of albumin.

The majority of these patients are malnourished—i.e., they are suffering from protein deficiency. This state is regularly accompanied by a reduction of basal metabolism (23 to 25). It has been tacitly assumed by some that oxidation is retarded by inhibition of pituitary activity when the supply of food becomes inadequate. The reduction of basal metabolism, according to this theory, arises from absence of thyrotrophic hormone. The striking similarity between the symptoms and signs of anorexia nervosa and those of Simmond's disease lends plausibility to this view (26). It might be inferred that both the reduction of basal metabolism and the serum iodine deficit of hypoalbuminemia are evidences of thyrotrophic inhibition in response to protein deficiency. If this were the case, similar phenomena should be observed in anorexia nervosa. In this peculiar state, however, the concentration of serum albumin is relatively well preserved. This is illustrated by the six cases presented in Table I. It will also be noted that serum precipitable iodine may remain within normal limits, although the basal metabolism is reduced to levels comparable to those seen in patients with myxedema. In only one of the six cases, B77426, is serum iodine definitely below normal limits. There is reason to question the diagnosis in this case on entirely independent grounds. The figures in P3027 are probably also below normal if consideration is given to the fact that they must be corrected for free iodine. The fact remains, however, that extreme malnutrition of anorexia nervosa with striking hypometabolism may be associated with normal serum albumin and serum precipitable iodine.

The discovery that hypoalbuminemia may be associated with serum iodine deficiency in the absence of definite evidence of hypothyroidism is a distinct challenge to the diagnostic value of the precipitable iodine method. Patients with low serum albumin have always presented a diagnostic problem. The basal metabolism, on which reliance has been generally placed, is of doubtful value in this state; serum cholesterol is equally unreliable. Reasons have been given for discounting these methods in the nephrotic syndrome. It cannot be assumed that the serum iodine deficiency is a sign of inactivation of the thyroid in response to malnutrition in view of the findings in anorexia nervosa. Indeed, the failure of serum albumin to

fall consistently in this disorder is one of a number of disturbing facts that may compel revision of the widely accepted theory that depletion of protein *per se* gives rise to hypoalbuminemia. The concentrations of precipitable iodine and of albumin in the sera of patients with authentic anterior pituitary deficiency have not been extensively investigated, as far as the authors are aware. Our own scanty data on the subject are presented in Table I. In one patient, B74923, with a chromophobe adenoma of the anterior pituitary with hypogonadism and extremely low urinary 17-ketosteroids, when the basal metabolism was -31 per cent the serum iodine was 3.6 γ per cent, serum albumin 4.49 per cent (Howe) and total cholesterol 206 mg. per cent. There was no evidence in serum electrolytes or chloride excretion of adrenal cortical deficiency. A patient, B76181, with a craniopharyngeoma and low urinary 17-ketosteroids, again without evidence of adrenal cortical insufficiency, had a basal metabolism of -28 per cent and serum precipitable iodine varying from 2.9 to 3.9 γ per cent. Serum albumin on one occasion was 3.62 per cent (Milne). Cholesterol fell from 229 to 146 mg. per cent under thyroid therapy, while serum precipitable iodine rose from 2.9 to 3.2 γ per cent. B69216, with a suprasellar tumor and hypogonadism, in addition to disturbances of the metabolism of bicarbonate and chloride indicative of adrenal cortical insufficiency, had a serum precipitable iodine of 1.7 γ per cent and a total cholesterol of 247 mg. per cent. In this case serum proteins were not measured. B98193, with presumably a chromophobe adenoma of the pituitary, had hypogonadism and adrenal cortical insufficiency. His serum albumin and cholesterol were normal, his precipitable iodine on one occasion low, on another within normal limits. If these cases are characteristic, hypopituitarism appears to lower basal metabolism and serum precipitable iodine, but not serum albumin nor cholesterol. The subject requires further investigation; but tentatively the effect of pituitary insufficiency on the composition of serum seems to differ from those of either anorexia nervosa or disorders attended by hypoalbuminemia. Among the features of hypopituitarism that require further inquiry is the apparent tolerance to thyroid developed by patients with these conditions. The iodine of the first three cases responded to thyroid medication

at first; but in each instance fell subsequently, although the dose of thyroid was maintained.

The clinical importance of the phenomenon can be best illustrated by two cases from the present series. The first, 576, Table I, has already been mentioned. The patient, a woman of 64, had profuse albuminuria with some evidences of arteriosclerosis and mild renal insufficiency, hypoalbuminemia of a severe grade, massive edema of the lower extremities and the lower two-thirds of the trunk, ascites and bilateral hydrothorax. A little earlier an operation had revealed a spontaneous cholecystocolostomy and a large gall-stone obstructing the colon. She had some hepatic insufficiency and bronchiectasis. She was extremely malnourished and able to eat little because of anorexia, nausea and occasional vomiting. Because of the dry, shrivelled appearance of her skin, scanty, falling hair and other stigmata suggestive of myxedema, serum precipitable iodine was measured, which proved to be only 1.3 γ per cent. Thyroid was, therefore, prescribed. A dose of 1 grain had no detectable effect. Even after she had received 3 grains daily for almost three weeks, the serum iodine was only 3.4 γ per cent. Finally, on 4 grains daily it rose to 5.0 γ per cent. The patient, however, wasted rapidly. Subsequently, when the dose of thyroid was reduced to 3 grains daily, the precipitable iodine fell to 2.8 γ per cent. It is, of course, impossible to say whether the large dose of thyroid accelerated wasting, in view of the numerous disorders from which she suffered. It was necessary, however, in order to bring her serum iodine into the lower normal range, to give as much thyroid as is usually required to induce hyperthyroidism and excessively high serum iodine in patients with true myxedema. Postmortem examination revealed, in addition to the fistula between the bile duct and the colon, chronic pyelonephritis, biliary cirrhosis and bronchiectasis, a thyroid gland that was somewhat hypoplastic, but had not the characteristic appearance of a myxedematous gland. It may well be that the low serum precipitable iodine was related only to the hypoalbuminemia in this case and that thyroid medication, by accelerating her metabolism, hastened her demise.

The second patient, 71570, a 59 year old male, was admitted to the hospital because of a subdeltoid

bursitis and a chronic infection of the left maxillary antrum. In addition he had been losing weight, strength and energy progressively for about four years. X-ray of the skull indicated that he had a craniopharyngeoma. He was wasted and had extreme anorexia. Blood sugar curves and studies of salt metabolism yielded no evidences of adrenal cortical insufficiency. Gonadal hypofunction was hard to evaluate in view of the undernutrition, but urinary 17-ketosteroids were distinctly reduced. His skin had a silky texture. Serum albumin and precipitable iodine were both low. At the same time the lipids were greatly reduced as they are in patients with malnutrition, in contrast to the normal values frequently encountered in pituitary insufficiency. On 2 grains of thyroid daily for two weeks, together with 30 mg. of methyltestosterone, the serum iodine did not rise; if anything, it fell. In this respect he differs sharply from the usual hypopituitary case. At the same time serum albumin diminished and his weight decreased. Again the question must be raised whether the low iodine in this case represented thyroid deficiency or was only associated with the hypoalbuminemia. The latter, in this case, was not so low as it was in the other cases with iodine deficits.

SUMMARY

In patients with profound hypoalbuminemia the precipitable iodine of the serum is often reduced to concentrations as low as those found in myxedema, without clinical evidence of thyroid deficiency. Administration of active thyroid substance in doses that are effective in the treatment of myxedema does not raise the precipitable iodine of these patients. Injections of enough salt-poor human albumin to raise the serum albumin of these patients considerably, does not consistently raise, and more often decreases, the precipitable iodine. The significance and clinical implications of the serum iodine deficiency are discussed.

Injection of enough salt-poor human albumin to raise the serum albumin of patients with hypoalbuminemia considerably causes the serum lipids to fall. This decrease occurs whether the lipids were originally high or low and affects all lipid fractions proportionally.

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NITROGEN BALANCE STUDIES ON THE KEMPNER RICE DIET

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Current interest in the dietary therapy of hypertensive states in man has been stimulated by Kempner's reports (1, 2, 3) of marked benefits achieved by his "rice diet." Dick and Schwartz (4) have reported that experimentally produced hypertension in dogs is favorably influenced by this regime, and Grollman and Harrison (5) have observed a significant reduction in the blood pressure of hypertensive rats fed only rice.

Kempner's "rice diet" (1, 2) is composed of rice, fruit, fruit juices and sugar, with vitamin and mineral supplements. He states: "Compared to diets previously used in hypertension, the rice-fruit-sugar diet is rigid. It contains in 2,000 calories about 5 gms. of fat and 20 gms. of protein derived from rice and fruit . . ." (2). Fluids are usually limited to 700-1,000 cc. of fruit juices per day. No added salt is permitted. Kempner further reports: "After two months of rice diet, the nitrogen excretion in the urine averaged 2.26 gm. per 1,000 cc., 2.26 gm. in 24 hours. If an allowance of 0.9 gm. per 24 hours is made for the excretion of nitrogen other than that excreted in the urine, the total nitrogen loss in 24 hours is about 3.16 gm. With a daily intake of $3.2 \times 6.25 = 20$ gm. of protein, these patients are in protein equilibrium" (1). No analyses of food or fecal nitrogen are reported. In all Kempner's papers it appears that the standard diet used by him contained an estimated 2,000 calories. In some cases where weight loss seemed excessive the caloric value of the diet was increased. However, in 11 of the 12 representative cases reported in his papers (1, 3) the daily caloric intake was 2,000 calories or less. In the 12th case, the intake was 2,400 calories. Body weights in these patients ranged from 54.0 to 93.0 kg., and no attempt was made to adjust intake to body weight or surface area.

Since Kempner's claim that nitrogen balance is achieved on such a low intake of protein was not in accord with other data (6, 7) study of the ni-

trogen balance of subjects on the "rice diet" was undertaken.

PROCEDURE

Six subjects were chosen for study. Four of these were psychotic adult males who showed no evidence of somatic disturbance and who were sufficiently in contact with reality to cooperate fully. The fifth subject was a patient with multiple sclerosis in remission. The sixth was a female nurse in good health who was in charge of the group throughout the day. The age, height, weight, surface area and basal caloric consumption of these subjects are given in Table I.

TABLE I
Physical characteristics and basal caloric expenditures

Subject	Age	Height	Weight	Surface area*	Basal† calories
		cm.	kg.	sq. m.	
1	25	162.5	89.4	1.92	—‡
2	40	177	64.5	1.77	1568
3	40	177	57.7	1.69	1451
4	31	174	73.3	1.85	1418
5	26	167.5	70.4	1.78	1604
6	25	175	86.9	2.00	1886
				aver. 1.84	aver. 1585
Hypertensive	27	170	74.5	1.85	2017

* Calculated according to the DuBois equation.

† Calculated from basal oxygen consumption determined with a McKesson Waterless Metabolizer, by multiplying the 24-hour oxygen consumption by 4.82.

‡ Repeatedly unsatisfactory.

A separate ward was set aside, and the group remained on this ward during the entire period. Reliable attendants, working in shifts, assured that the ward was continuously supervised. The subjects were ambulatory but engaged only in ward activities. All food was specially weighed and prepared in a separate diet kitchen, and no edibles other than the diet were allowed on the ward. The latrine was kept locked, and the male subjects could enter only with an attendant. This permitted rigid control of the collection of urine and feces.

The regular hospital diet, containing approximately 80 gm. of protein, was replaced by a protein-depletion diet. This diet was calculated to contain approximately 1,714 calories, with 8.4 gm. of protein (Table II-A). At the end of a four-day period on this depletion diet, the "rice diet" was introduced and was continued for eight days.

The diet consisted of 200 gm. of rice (dry weight) with additional calories supplied by fruit, sugar and fruit juices to a total of approximately 2,289 calories per day. This provided an estimated intake of 21 gm. of protein. The only fluids permitted were the 1,000 cc. of fruit juice each day. No salt was allowed. Daily vitamin and mineral supplements were provided (Table II-B). This diet differs from the one used by Kempner in his nitrogen balance studies only in having a somewhat higher caloric value achieved by the use of a larger quantity of sucrose.

All subjects consumed the total diet each day. Twenty-four-hour urine collections were made in jars containing 5 cc. of 50 per cent nitrogen-free sulfuric acid, and aliquots were stored in a refrigerator. Feces collected throughout the eight-day period on the "rice diet" were likewise refrigerated. Carmine was used to mark the beginning and end of the collection period.

Aliquots of the 24-hour urine collection of the last day on the regular hospital diet, the final day of the depletion period, and the third, sixth and eighth days on the Kempner regime were analyzed for nitrogen. The feces for the eight-day period on the "rice diet" were pooled and homogenized in a Waring Blender, and an aliquot was analyzed for nitrogen. Diets were likewise homogenized before analysis. Nitrogen determinations were carried out by a Macro-Kjeldahl method, using the Arnold-Gunning digestion mixture and Meeker's boric acid modification for distillation (8). All analyses were made in duplicate.

A patient with severe hypertension was placed on the "rice diet" for a period of 90 days and his nitrogen bal-

TABLE II-A
Depletion diet

	Weight	Calculated values	
		Protein	Calories
	gm.	gm.	
Cornmeal	15, dry	1.3	53
Fruits, peaches, canned	100	.5	102
Pineapple, canned	100	.4	87
Apple, baked	115	.5	181
Vegetables, tomatoes, canned	80	.8	16
Carrots, canned	100	1.0	37
Celery	25	.35	6
Asparagus, canned	50	.9	10
String beans, canned	50	1.0	18
Turnip	50	.55	17
Cabbage	50	.7	15
Bouillon	1 cube	.4	3
		8.4	545
Gingerale 24 oz.	720	—	258
Coca Cola 24 oz.	720	—	258
Sucrose*	165	—	653
Total		8.4†	1714

* Caloric value calculated from data in Handbook of Physics and Chemistry, Chemical Rubber Publishing Co., Cleveland, 1947.

† By analysis 7.03 gm.

TABLE II-B

Rice diet

	Weight	Calculated values	
		Protein	Calories
	gm.	gm.	
Rice	200, dry	15.4*	700
Juices, orange	200	1.2	80
Pineapple	400	1.2	216
Grapefruit	400	1.3	160
Fruits, apricots, canned	130	.8	119
Applesauce	200	.4	160
Pears, canned	200	.4	150
Raisins	15	.3	45
Sucrose†	166.5	—	659
Total		21.0†	2289

* By analysis 12.0 gm.

† Caloric value calculated from data in Handbook of Physics and Chemistry, Chemical Rubber Publishing Co., Cleveland, 1947.

‡ By analysis 16.43 gm.

Vitamin and mineral supplement

Ferrous Sulfate, 0.6 gm.; Vitamin A, 17,000 U.S.P. units; Vitamin D, 1,600 U.S.P. units; Thiamin Hydrochloride, 5 mg.; Riboflavin, 5 mg.; Calcium Pantothenate, 10 mg.; Nicotinamide, 30 mg.; Ascorbic Acid, 100 mg.

ance also was studied. Physical characteristics of this patient are presented in Table I.

Cholesterol determinations were carried out by the method of Bloor (9). Hematocrit was determined in Wintrobe tubes after centrifuging for 30 minutes at 2,500 r.p.m. Whole blood hemoglobin was determined by the direct photometric method of Sanford, Sheard and Osterberg (10). The method of Phillips *et al.* (11) was used for determining total serum protein. Non-protein nitrogen determinations were carried out by the method of Folin and Wu (12). Basal caloric consumptions were determined by the use of the McKesson Waterless Metabolor and the basal metabolic rates calculated according to the standards of Aub and DuBois.

RESULTS

Kjeldahl analysis revealed the nitrogen content of the "rice diet" to be 2.63 gm. (16.43 gm. protein) (Table II-B), a value 22 per cent lower than that calculated from tables (13). This was due largely to the fact that the protein content of the rice (an ordinary commercial milled type) was 6.0 per cent by analysis rather than the estimated 7.7 per cent.

The urinary nitrogen excretion of the six subjects on the final day of the hospital diet, the final day of the depletion diet, and the third, sixth and eighth days of the "rice diet" are shown in Table III. The average urinary nitrogen excretion on

TABLE III
Nitrogen excretion studies

Standard diet	Depletion diet	Rice diet				Rice diet		
Urine N/24 hrs.	Urine N/24 hrs.	Urine N/24 hrs.				Fecal N/24 hrs.	Total final output/24 hrs.	N balance* final 24 hrs.
	4th day	3rd day	6th day	8th day				
gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1. 16.65	4.51	Menstruating	3.70	3.46	1.09	4.55	—1.92	
2. 9.59	4.26	4.72	3.78	3.10	.79	3.89	—1.26	
3. 10.62	5.38	4.90	4.50	4.33	1.60	5.93	—3.30	
4. 11.02	6.93	4.20	4.20	6.02	1.37	7.39	—4.76	
5. 15.74	7.80	5.90	6.64	5.87	1.06	6.93	—4.30	
6. 14.86	6.80	6.03	6.34	5.27	1.08	6.35	—3.72	
Av. 13.08	5.95	5.15	4.86	4.68	1.17	5.85	—3.22	

* N intake in all patients 2.63 gm./24 hours.

the eighth day of the "rice diet" was 4.68 gm. per 24 hours.¹ The average daily fecal nitrogen excretion was 1.17 gm. The total nitrogen output (urinary plus fecal) for the final day on the diet averaged 5.85 gm. Thus nitrogen excretion exceeded nitrogen intake by 3.22 gm. per 24 hours.

The data on the hypertensive patient studied for 90 days are presented in Table IV. On the 90th day, urinary nitrogen excretion was 3.81 gm. The average daily fecal nitrogen excretion during the

TABLE IV
Hypertensive patient
90th day of rice diet

Urine N/24 hours	3.81 gm.*
Feces N/24 hours	1.49
Total N/24 hours	5.30
N balance	—2.67

*30th day—4.93
60th day—4.64

	Initial	Final	Change
Weight, kgs.	71.7	59	—12.7
N.P.N., mg./100 cc.	41	28	—13
	220 280	140 160	
Blood pressure, mm. Hg	130 190	100 115	
B.M.R.	+13	+4	—9
Basal calories	2012	1663	—349
Total protein, gm./100 cc.	7.2	6.1	—9

¹ Ten representative urine specimens also were analyzed in the laboratory of the Department of Nutrition, Harvard School of Public Health. The results were in agreement within 2 per cent of our data. We wish to thank Dr. D. M. Hegsted for his kind cooperation.

final week was 1.49 gm. Thus total nitrogen excretion was 5.3 gm. per day. In this patient, nitrogen excretion exceeded intake by 2.67 gm. per day.

Table V presents the data on blood pressure, body weight and basal metabolic rate. It will be noted that the subjects, all of whom were initially normotensive, showed, without exception, no significant change in blood pressure. Weight loss on the "rice diet" varied from 1.6 to 3.0 kg. with an average loss of 2.1 kg. during the eight-day period. No significant change occurred in the basal metabolic rate nor were there any appreciable changes in temperature, pulse and respiration throughout the course of the experiment except in subject number 4 who suffered an acute upper respiratory infection with fever during the fifth

TABLE V
Changes in blood pressure, weight and basal metabolic rate on rice diet

Subject	Blood pressure		Body weight			Basal metabolic rate		
	Before	8th day	Before	8th day	Change	Before	8th day	Change
			kg.	kg.	kg.			
1	114/76	118/72	89.4	87.4	—2.0	*	*	*
2	104/68	100/70	64.5	62.9	—1.6	—5	—21	—16
3	128/74	122/72	57.7	56.1	—1.6	—8	+2	+10
4	128/78	128/92	73.3	70.8	—2.5	—6	—6	0
5	116/78	108/72	70.4	68.5	—1.9	—10	—9	+1
6	130/90	132/82	86.9	83.9	—3.0	—4	—16	—12

* Repeatedly unsatisfactory.

to seventh days of the "rice diet." This infection coincided with a rise in his nitrogen excretion.

In Table VI are given the data on blood constituents. All subjects showed a decrease in serum cholesterol with an average fall of 44 mg. per 100 cc. Hemoglobin, hematocrit, and total protein showed a slight increase. The non-protein nitrogen was decreased in three subjects.

DISCUSSION

It is well known that dietary requirements vary with body size. The minimal daily nitrogen requirement for man on a mixed diet has been reported to be approximately 2.9 gm. per sq. m. of body surface (6, 7) or 5.2 gm. nitrogen (32.5 gm. protein) for a man weighing 70 kg. and with a surface area of 1.8 sq. m. Calculated on this

TABLE VI
Blood changes on rice diet

	Cholesterol mg./100 cc.			Total protein gm./100 cc.			N.P.N. mg./100 cc.			Hemoglobin gm./100 cc.			Hematocrit per cent		
	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
1	165	150	-15	7.8	8.3	+0.5	38	29	-9	14.2	15	+0.8	45	46	+1
2	255	225	-30	8.3	9.2	+0.9	39	39	0	15.8	16	+0.2	46	49	+3
3	275	175	-100	5.9	7.8	+1.9	41	32	-9	15.6	15.8	+0.2	50	50	0
4	200	160	-40	8.2	7.1	-1.1	39	29	-10	14.6	14.2	-0.4	43	44	+1
5	185	130	-55	8.1	9.2	+1.1	35	35	0	14.2	15.4	+1.2	43	48	+5
6	215	190	-25	7.5	9.1	+1.6	33	36	+3	15.8	16.2	+0.4	48	51	+3
Av.	216	172	-44	7.6	8.4	+0.8	38	33	-5	15.0	15.4	+0.4	46	48	+2

basis, our subjects, with surface areas ranging from 1.69 to 2.00 sq. m., would require 4.87 to 5.80 gm. nitrogen (average 5.39) in a mixed diet.

For an average man (70 kg., 1.8 sq. m.) on a diet containing only vegetable protein the minimal nitrogen requirement has been found to be 4.8 to 7.9 gm. per day (29.7 - 49.1 gm. protein) depending upon the protein used (6, 7). With diets containing rice as the major protein source the minimal requirement is even higher. Aron and Hocson (14) and Kumagawa (15) studying such diets reported that nitrogen balance could be achieved only when the daily nitrogen intake was no less than 0.18 gm. per kg. body weight, or more than 12 gm. for a 70 kg. man.

Urinary nitrogen excretion values as low as the 2.26 gm. per day reported by Kempner on the "rice diet" have been reported by other workers only on nitrogen-free, high caloric regimes (16, 17). On a nitrogen-free, high caloric diet the nitrogen excretion represents endogenous protein breakdown. The value obtained is a function of the energy metabolism and is generally accepted to be approximately 2.0 mg. nitrogen per basal calorie (18, 19, 20). The average basal caloric expenditure for adult males in the age group 20 to 40 is 39.5 calories per sq. m. per hour (21). For an average male with a surface area of 1.8 sq. m., the daily basal caloric expenditure is 1,706 calories. At this metabolic level, the calculated endogenous nitrogen on the basis of 2 mg. per basal calorie is 3.4 gm. per day. In our five male normotensive subjects with an average surface area of 1.82 sq. m. and an average basal caloric expenditure of 1,585 calories, the calculated endogenous nitrogen metabolism is 3.17 gm. Bricker *et al.* (6) have recently reported excretions of

1.4 mg. per basal calorie. This figure would lower the calculated endogenous nitrogen metabolism for our subjects to 2.22 gm. per day, but it must be pointed out that studies on diets containing protein have not yielded such low figures (6, 7). In order to achieve balance, nitrogen intake must always be in excess of the endogenous nitrogen breakdown due to the fact that dietary proteins are not completely digested and utilized.

It is possible that even with the provision of 2,300 calories in our diet rather than the 2,000 calories used by Kempner, the caloric requirements of all of our subjects were not satisfied. The negative nitrogen balance, therefore, may be due at least in part to an inadequate caloric intake. It appears likely, however, that at least in those subjects with basal caloric expenditures of 1,418, 1,451 and 1,568 calories the caloric intake was adequate.

The loss of weight in all subjects cannot be completely accounted for by the negative nitrogen balance. The restricted salt and fluid intake were undoubtedly contributory factors. A third factor to be considered is the possible inadequacy of caloric intake. A similar excessive loss of weight has been noted by Eckhardt *et al.* (22) in subjects with adequate caloric intake but deficient protein intake.

It has been shown that on low protein test diets minimal urinary nitrogen excretion levels may be closely approached in three to four days, particularly if a preliminary protein depletion diet is employed (7, 23, 24). After four days on the protein depletion diet and eight days on the "rice diet," all of our subjects were in negative nitrogen balance with a daily average excess of excretion over intake of 3.22 gm. Although the data on urinary nitrogen excretion suggest that

the minimal level of excretion had not been achieved, the slope of the excretion curve indicates that this level was closely approximated (Figure 1). It thus seems unlikely that nitrogen balance can be attained on a Kempner regime providing 3.2 gm. nitrogen (20 gm. protein) per day. Even after 90 days on the "rice diet," the hypertensive patient showed essentially the same order of magnitude of negative nitrogen balance (2.67 gm. per day) as was shown for the subjects in the shorter study. This patient had, at the start of the study, a basal caloric expenditure of 2,012 calories and lost weight rapidly for seven weeks (10 kg.). The weight loss during the last six weeks of the study was 2.7 kg. At the time of final balance

studies, the basal caloric expenditure was 1,663 calories.

There is no evidence to indicate that the "rice diet" has any unusual ability to satisfy the nitrogen demands of the body. In order to achieve nitrogen balance, it appears that nitrogen intake must be as high on this diet as on other diets consisting mainly of vegetable protein.

It is worthy of note that the hypertensive patient showed a marked clinical improvement on the Kempner regime although he failed to maintain nitrogen equilibrium. During the diet therapy, there was a significant drop in blood pressure and return to upright of T-waves in the electrocardiogram. No fresh retinal hemorrhages oc-

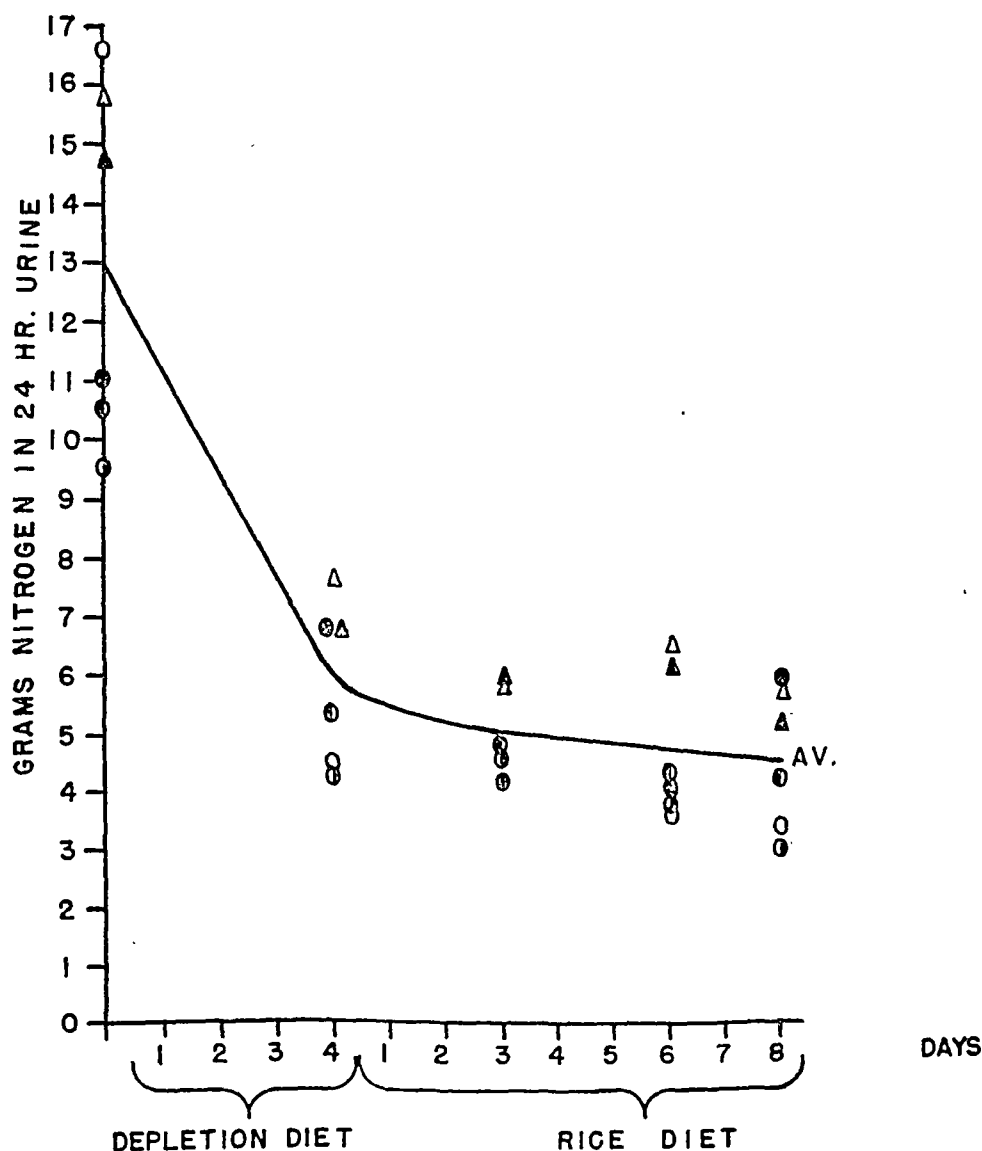


FIG. 1. URINARY NITROGEN EXCRETIONS OF SIX SUBJECTS ON THE PROTEIN DEPLETION AND RICE DIETS

Each subject is represented by a separate symbol.

curred, and there was dramatic symptomatic relief of headaches, dizziness and malaise.

SUMMARY AND CONCLUSIONS

1. Nitrogen balance was studied in six normotensive subjects on the Kempner "rice diet," which, by analysis, was found to contain 2.63 gm. nitrogen per day.

2. After eight days on the "rice diet" (preceded by four days on a protein depletion diet) analyses of food, urinary and fecal nitrogen revealed that all subjects were in negative nitrogen balance. The excretion was greater than the intake by an average of 3.22 gm. nitrogen per day.

3. An additional subject, suffering from hypertension, was followed for 90 days on the "rice diet" and showed a negative nitrogen balance of 2.67 gm. per day.

4. The "rice diet" shows no unusual ability to satisfy the nitrogen requirements of the body.

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THE EFFECTS OF THE RATE OF ADMINISTRATION OF AMINO ACID PREPARATIONS ON URINARY WASTAGE OF AMINO ACID NITROGEN IN MAN

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One of the major objections to the clinical use of mixtures of amino acids derived from hydrolysis of casein is the occurrence of nausea, vomiting, flushing, and headaches. These undesirable symptoms occur more frequently if the rate of administration is rapid. In a previous communication (1) it was shown that the elimination of many of these reactions could be accomplished by reducing the rate of infusion; it was further observed that mixtures of amino acids could be given at exceedingly rapid rates (100 ml. of a 10 per cent mixture per minute or the equivalent of 100 grams of protein could be given intravenously in less than three hours) without causing any discomfort to the patient.

Earlier investigators (2, 3, 4) found that if amino acid preparations were given intravenously at a slow rate, usually less than 8 per cent of the administered amino acid nitrogen was lost by urinary spillage. Thus, it was desirable to learn whether the advantages gained by the rapid intravenous infusion of the well-tolerated amino acid mixtures might be offset by an increased urinary loss. This study was planned to compare the urinary amino acid nitrogen loss following the intravenous administration of amino acid mixtures at rapid rates with the loss which occurred following the intravenous injection of casein digests at rates approaching maximum tolerance. In addition, studies were designed to obtain comparable control data regarding the amount of amino acid nitrogen lost due to the "washing-out" effect following the injection of equal volumes of physiological saline.

METHODS

Male patients with no detectable metabolic disorders were used as subjects for this study. All food was withheld after the previous evening meal until the four-hour study period of the following morning was completed. On the morning of the day of the study the patient

emptied his bladder and the intravenous injection of the test solution was started; all voided urine was collected for the next four hours with the patient emptying his bladder at the end of the period. The same method for urine sample collection was used with each preparation studied. The interval between each test for a given patient varied from three to five days, thus providing a period for stabilization. By using a urine collection period of only four hours an important practical advantage was gained in that there was only a slight interference with the general hospital routine; the patient lost only one meal (breakfast) and was therefore more cooperative during the entire period of study.

Three amino acid preparations were used: Preparation I¹,² consisted of a 10 per cent solution of an enzymatic hydrolysate of casein, Preparation II³ was an acid hydrolysate of casein fortified with tryptophane, and Preparation V⁴ was a "VUJN" type of mixture of amino acids prepared by the recombination and fortification of fractions of a casein hydrolysate. All amino acid solutions used were diluted with sterile water to provide a test dose which had a total nitrogen content of approximately 5.4 gms. per 500 ml.

The first group of five patients received in successive periods of study all three amino acid preparations and the physiological saline control solution at a constant rate of 500 ml. in two hours. The second group of ten patients received the test dose of the three amino acid preparations at different rates of injection: Preparation I was given in one and one-half hours, Preparation II in two hours and Preparation V in one hour. The amino acid preparations in this phase of the study were administered at rates which previous experience had shown approached the maximum tolerated rate in the majority of the patients. A third group of four patients each received Preparation V administered first at a rate of

¹ Preparation I (Amigen, 10 per cent solution) was supplied by Mead Johnson and Company, Evansville, Indiana.

² The numbers designating the preparations are the same which have been used in previous reports from this laboratory.

³ Preparation II (Parenamine 15 per cent) was supplied by Frederick Stearns and Company, Detroit, Michigan.

⁴ Preparation V (VUJN-IX) was supplied by Merck and Company, Inc., Rahway, New Jersey.

500 ml. in two hours and subsequently the same amount in 35 minutes.

The amino acid nitrogen content in the urine was estimated by the copper method of Albanese and Irby (5). Free amino acid nitrogen was determined by doing the analysis directly on the urine. Total amino acid nitrogen was determined in the urine samples after hydrolysis which was accomplished by mixing 20 cc. of urine with 5 cc. of concentrated HCl and autoclaving at 120° C. for five hours. The difference between the total and the free amino acid nitrogen was considered to be "bound" amino acid nitrogen.

RESULTS

The results of the comparison of the urinary losses of amino acid nitrogen as initially observed by infusing all three of the preparations studied at the same rate and also at the same total nitrogen level are summarized in Figure 1. In addition to the amino acid preparations, these patients subsequently received an infusion of physiological saline at the same rate, the amino acid nitrogen lost in the urine is also presented in this same figure. Under the conditions of the experiment, Prepara-

tion I caused the largest amount of total amino acid nitrogen to appear in the urine; variations in the amounts of total amino acid nitrogen excreted during the four-hour test period were great, the extremes being 300 and 900 mg., with an approximate average of 550 mg. The increased loss of total amino acid nitrogen appeared to be due mainly to "bound" amino acid nitrogen, since the loss of free amino acid nitrogen was of the same magnitude as with the other two preparations tested. The amount of "bound" amino acid nitrogen excreted after the infusion of Preparation I was approximately equal to the total amino acid nitrogen excretion with the other two preparations. The amount of "bound" nitrogen excreted following the administration of either Preparation II or V was of the same general magnitude as that found in the urine after the saline infusion. Following the administration of Preparation II an average of about 400 mg. of total amino acid nitrogen appeared in the urine during the four-hour period; the amounts of free and bound amino acid nitrogen

FOUR HOUR URINARY AMINO ACID NITROGEN EXCRETION
FOLLOWING 5.4 GM. NITROGEN INFUSION*

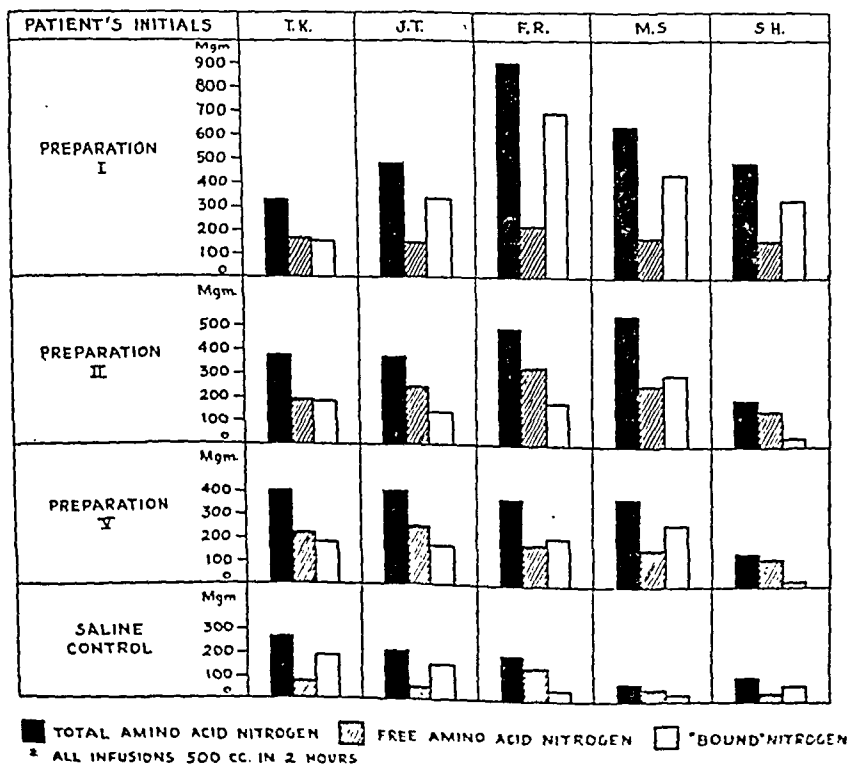


FIG. 1

FOUR HOUR URINARY AMINO ACID NITROGEN EXCRETION
FOLLOWING 5.4 GM. NITROGEN INFUSION *

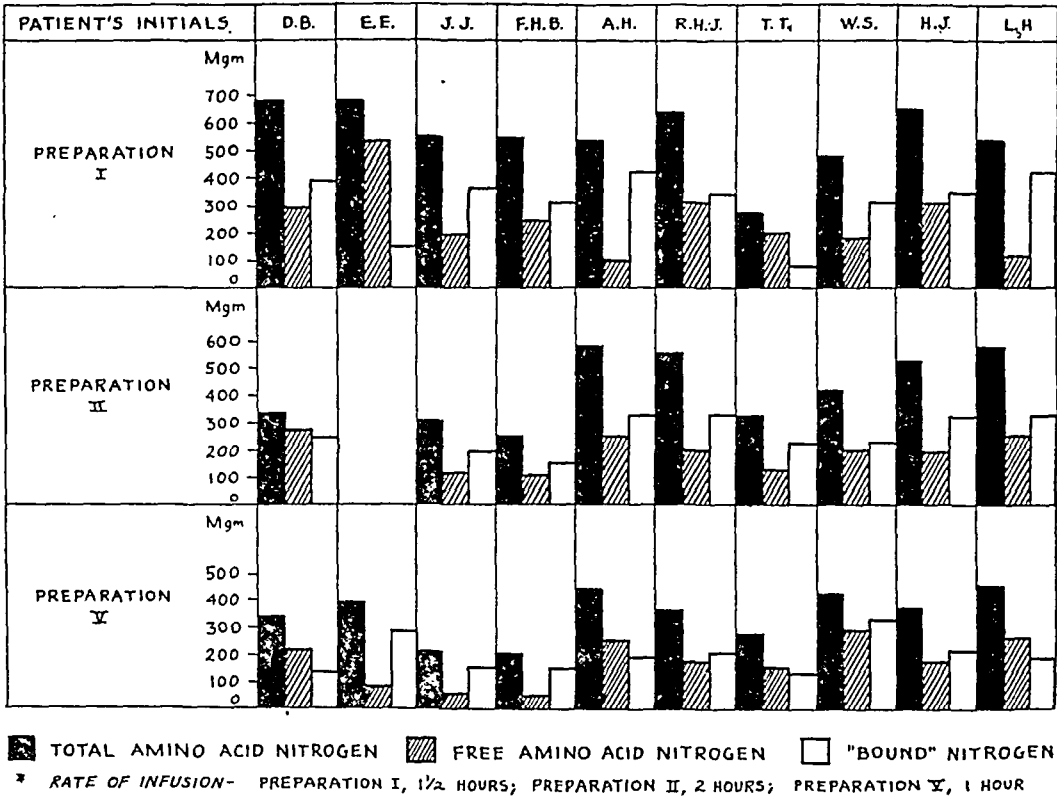


FIG. 2

were almost equal in the five patients studied. The amino acid excretion pattern following the administration of Preparation V was in general similar to that of Preparation II, approximately 400 mg. of amino acid nitrogen being lost.

If the amount of total amino acid nitrogen excreted following the saline infusion were subtracted from the total amino acid wastage, which followed the administration of Preparation II or V, there would be little nitrogen lost during the four-hour test period, with subject M. S. being an exception. This becomes evident if a vertical comparison for any one subject is made of the data in Figure 1. If a comparison is made of the free amino acid nitrogen lost following the administration of all three preparations, the small amounts and the relative constancy of the free amino acid excretion are striking.

Since both the amount of total amino acid nitrogen administered to subjects and the amount of total amino acid nitrogen excreted due to the infusion were known, it was possible to determine the per cent of administered total amino acid nitrogen wasted due to urinary spillage. This is

summarized in Table I. The per cent of administered total amino acid nitrogen of Preparation I lost ranged between 1.6 and 19.7. The amount of Preparations II and V lost under similar conditions was less variable and usually ranged about three to five per cent.

A study of the urinary wastage of amino acid nitrogen after intravenous administration of the three preparations investigated given at different rates is presented in Figure 2. It should be noted that Preparation I was administered in a one and one-half hour period, while Preparation II was administered in a two-hour period, and Preparation V was injected in the short period of only one hour. It is of interest that usually less total amino acid nitrogen was lost in the urine after the administration of Preparation V than with other preparations tested, although the speed of its administration was greatest. In only one case (T. T.) was more amino acid nitrogen lost after the administration of Preparation V than with either of the other products.

Since it was previously shown that an amino acid mixture (Preparation V) could be tolerated

well at exceedingly rapid rates of administration (1), the effect of rapid infusions of this preparation on the urinary wastage of amino acid nitrogen was investigated more intensively (Figure 3). Four subjects received Preparation V intravenously in a two-hour period and the urinary wastage of amino acid nitrogen was determined. Subsequently, after a three- to five-day period, these same four subjects received 500 ml. of this same amino acid mixture in a 35-minute infusion. In three of the four patients studied, there was less amino acid nitrogen spilled in the urine following the rapid rate than at the slower rate of infusion.

DISCUSSION

No direct comparison of the excretion values presented in this study with those available in the literature is possible because the periods of urine collection are different. However, in general, the amounts of amino acid nitrogen lost in the four-hour period are comparable to the values reported in the literature for periods from 12 to 24 hours. It would appear that the major wastage occurs during the first few hours following the intravenous administration.

By determining the amount of amino acid nitrogen in the urine following the administration of physiological saline in a given patient, it was possible to estimate the amino acid wastage in that pa-

tient due to the "washing-out" effect produced by fluid containing no nitrogen. In considering the data present in Table I, it should be noted that the amount of amino acid nitrogen lost in the urine was calculated as the difference between the amount lost in the four hours following the beginning of the amino acid infusion and the amount lost in a similar period following a saline injection. These values represent, therefore, the amino acid wastage due only to the supplementary alimentation.

TABLE I

The per cent of administered total amino acid nitrogen excretion in a four-hour period after giving the various preparations intravenously

The amino acid nitrogen losses following the physiological saline infusion have already been subtracted from the values in the table.

Patients' initials	T. K.	J. T.	F. R.	M. S.	S. H.
Preparation I	1.6	7.6	19.7	14.9	10.4
Preparation II	3.4	5.1	9.4	13.7	1.9
Preparation V	3.5	4.8	4.3	7.5	0.9

The excretion of total amino acid nitrogen after the administration of Preparation I was greater than with the other preparations tested. From the data in Figure 1 it is noted that there were large increases in "bound" nitrogen; furthermore, this increased loss of "bound" nitrogen is sufficient to account for the large loss of total amino

FOUR HOUR URINARY AMINO ACID NITROGEN EXCRETION
FOLLOWING 5.4 GM. NITROGEN INFUSION
PREPARATION V

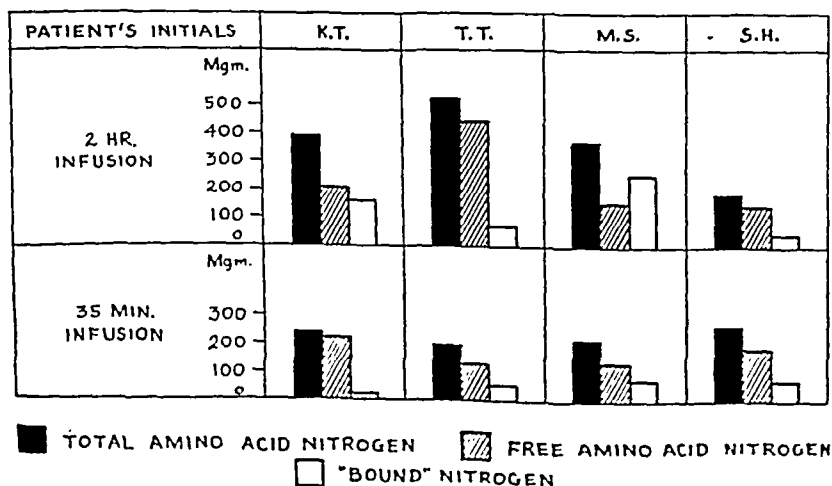


FIG. 3

acid nitrogen. Christensen and co-workers (6) have reported that human subjects have a limited ability to retain peptides, for after the administration of an enzymatic hydrolysate of casein (Amigen) the peptides were rapidly excreted by the kidney without a measurable quantity being present in the blood stream. Christensen also concluded that these observations indicate that the tissue utilization of peptide nitrogen is definitely limited. Increased urinary loss of "bound" amino acid nitrogen has also been found to occur in dogs after the intravenous administration of Amigen by Cox and Mueller (7). Recently, Christensen and associates (8) have reported observations concerning the urinary loss of "bound" amino acid nitrogen following the intravenous administration of two different peptide-containing protein hydrolysates and stated that the "bound" amino acid nitrogen in a fibrin hydrolysate was better utilized than that present in an enzymatic hydrolysate of casein. These workers proposed a new concept, suggesting that large urinary losses of "bound" amino acid nitrogen following intravenous administration of a partial hydrolysate of protein may offer the possibility of serious losses, either of a qualitative or quantitative nature, of the injected amino acids.

Preparation V had the marked clinical advantage in that it could be given at exceedingly rapid rates without producing ill effects. Yet, in spite of this rapid rate of infusion, approximately 15 ml. per minute, there was little or no increase in the wastage of amino acid nitrogen as measured by an increase in urinary amino acid nitrogen. Silber, Seeler, and Howe (4), working with dogs, have shown that doubling the rate of administration of a preparation similar to Preparation V, from 6 to 12 mg. of nitrogen per Kg. per minute did not increase the rate of urinary loss of amino acid nitrogen. The work presented here, using human subjects and a rapid administration of amino acids (Preparation V), is in agreement with their report. It is rather striking that so little free amino acid nitrogen is lost after the intravenous administration of any of the preparations tested as compared to the amount of amino acid nitrogen administered. This is true, regardless of the speed of infusion, providing the material is well tolerated. From the data presented in the four-hour test period, it appears that the human organism holds

tenaciously to amino acid nitrogen given intravenously.

Individual variation of patients was rather great in regard to the urinary excretion of amino acid nitrogen. This is illustrated in Table I where patient M. S. had high urinary losses of amino acid nitrogen regardless of the preparation given. Inspection of the total amino acid nitrogen excretions given in Figure 2 further stresses this point.

One of the major purposes of this work was to determine whether with rapid rates there was increased spillage of the amino acid preparation into the urine. From the data presented, it may be concluded that there is little if any increased amino acid wastage due to the rapid rate of administration of the VUJN type of mixture of amino acids.

SUMMARY

A comparison was made in 15 human subjects of the urinary loss of amino acid nitrogen in a four-hour period following the administration of three different amino acid preparations, each containing 5.4 gm. of nitrogen. In addition, in five of these patients the amino acid nitrogen content of the urine was determined for the same period after an infusion of physiological saline. The injections of the protein hydrolysates were given at rates which previous experience had shown to approach the maximum tolerated rate in the majority of the patients.

The greatest urinary wastage of amino acid nitrogen occurred with the amino acid solutions prepared by the enzymatic hydrolysis of casein, a preparation containing large amounts of peptide nitrogen. The increased loss of total amino acid nitrogen which followed the use of this digest appeared to be due mainly to "bound" amino acid nitrogen, since the loss of free amino acid nitrogen was of the same magnitude as with the other preparations tested.

The per cent of the total administered amino acid nitrogen lost in the urine in a four-hour period following the enzymic hydrolysate of casein ranged between 1.6 and 19.7 per cent. Under similar conditions, usually 3 to 5 per cent of the total amino acid nitrogen was found in the urine after the administration of either an acid hydrolysate of casein or a mixture of amino acids. In contrast,

there was little free amino acid nitrogen lost after the administration of any of the preparations studied regardless of the speed of infusion. Mixtures of amino acids, if well tolerated, can be given at exceedingly rapid rates without causing increased spillage into the urine.

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THE COMPARATIVE EFFECTS OF CONTINUOUS AND INTERMITTENT PENICILLIN THERAPY ON THE FORMATION OF ANTISTREPTOLYSIN IN HEMOLYTIC STREPTOCOCCAL PHARYNGITIS¹

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In the Spring of 1947 an epidemic of streptococcal pharyngitis and scarlet fever attacked a large permanent army post⁵ numbering about eight thousand troops. A carrier survey just prior to this outbreak had disclosed a high rate (17 per cent) of carriers of beta hemolytic streptococci. Study of these organisms and those isolated from patients during the epidemic revealed that all were of Lancefield Group A. Seven-eighths of those isolated from carriers, and all streptococci isolated from patients, were either type 23 or 19. Infections caused by type 23 predominated and constituted about 70 per cent of all cases.

During a 60-day period, 184 patients received a preliminary diagnosis of streptococcal pharyngitis or scarlet fever and were placed in a special study group at the time of their admission to the post station hospital.

The objectives of this study were: (1) to evaluate and delineate the natural history of streptococcal pharyngitis in the young male, (2) to assess the value of three treatment schedules (detailed below), and (3) to determine and evaluate the antistreptolysin antibody response of patients in each of the three treatment groups.

This paper will be concerned only with the third objective of this study.

OUTLINE OF THE INVESTIGATION

Laboratory methods

The following studies were made of each patient, usually within 24 hours of admission: (1) Total leukocyte

¹ The authors wish to acknowledge their indebtedness to M/Sgt. R. J. Helmold who supervised the antistreptolysin titration and to Mrs. Florence Kessler, Bacteriologist, for their invaluable cooperation in this study.

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count, (2) urinalysis, (3) chest X-ray, (4) Dick test, (5) antistreptolysin titer, and (6) throat culture.

Subsequently, the total leukocyte counts were repeated on the fourth and ninth days; urinalysis and Dick test were repeated on the ninth day; throat cultures were repeated on the fourth, ninth, and 21st days, and the antistreptolysin titer was determined on the 21st day following admission.

Throat cultures were obtained as single specimens without the use of special methods or media. A cotton swab was touched to the tonsils and posterior pharynx, then immersed in brain-heart infusion broth, where it remained at room temperature until streaked over horse blood agar. After incubation, plates were studied with respect to the proportion of colonies producing beta hemolysis. No detailed colony counts were made, but all plates were evaluated by the same experienced bacteriologist with respect to predominance of beta hemolytic organisms. Cultures were reported as showing "predominating," "mixed," or "no significant" growth on blood agar. Organisms showing beta hemolysis on the first and final cultures were isolated in pure culture and forwarded to the First Army Laboratory for grouping and typing.

Antistreptolysin titration was performed by M/Sgt. R. J. Helmold using a modification of Todd's (1) original method. This modification entails the addition of 50 cc. of a Dextrose-Phosphate buffer to each liter of sterile Todd-Hewitt broth, and the use of M/25 Cystiene HCl as a streptolysin reducing agent (added to the streptolysin just prior to its use). A two-tube rise in titer has been regarded as significant. A fall in titer has not been accepted as evidence of a recent infection.

Preliminary selection of patients

During the period of study one or both of the authors personally examined in the hospital receiving office virtually all patients admitted with fever or complaints referable to the upper respiratory tract. Those suspected of streptococcal pharyngitis on the basis of previously defined clinical criteria were dispatched in approximate alternate rotation to one of several "study wards."

Although the work of the Commission on Acute Respiratory Disease (2) has emphasized the difficulty of establishing a clinical diagnosis of streptococcal pharyngitis, Rantz, *et al.* (3) have stated that such a diagnosis may be made with reasonable accuracy. With the use of criteria similar to those of Rantz, an attempt was made to

differentiate streptococcal sore throat from "non-specific pharyngitis" during a period when 516 cases of upper respiratory infection were admitted to the hospital. Of this number, 184 were separated from the others by the following criteria:

Clinical criteria

(1) *History*: The patients usually gave a history of the rapid onset of malaise, sore throat, chilliness or shivering chills. Vomiting, moderate prostration, and generalized aching were frequently present.

(2) *Physical signs*: The pharynx, tonsils and palate were invariably red and edematous, although the edema was often slight. Edema of the uvula proved almost pathognomonic. The soreness of the throat was often evident in the cautious manner in which the patient opened his mouth. The presence of exudate had been anticipated as a diagnostic key, but this finding was often absent. When present, it was green and confluent. Tender cervical adenitis, especially of the tonsillar nodes, was sought for and was usually evident.

The oral temperature was usually at least 100 degrees F. and prostration was sometimes severe.

A frank scarlatinal rash was of obvious diagnostic value and was considered indicative of streptococcal infection when present.

SEPARATION AND TREATMENT OF PATIENTS

At the commencement of the study, virtually all of 54 patients selected by these clinical criteria were treated with customary penicillin dosage, i.e., 20-50,000 units every three hours for a period of four to seven days (this schedule is hereafter referred to as Penicillin II). Subsequently, however, the remaining patients (130) were assigned alternately to one of two study wards; in one, antimicrobial therapy was administered and in the other no specific treatment was given. With the exception of two patients suffering from peritonsillar abscess at the time of admission, separation of patients into "treated" and "untreated" groups was effected *without regard for the severity of the disease*. Ward officers on the "untreated" wards were not permitted to initiate penicillin therapy without consulting one of the investigators. This point is stressed because in the available literature on the therapy of streptococcal pharyngitis the establishment of a large, strict control group, untreated with sulfonamides or salicylates, has not been reported. Most authors acknowledge that therapy was usually given the most severely ill, and that such therapy was often at the discretion of ward surgeons.

"Untreated" group

The patients in this group received symptomatic therapy in the form of hot saline gargles or irrigations, obligatory bed rest for three days, and codeine when necessary. Salicylates were specifically interdicted in order to permit accurate observation of fever duration. All patients were closely watched for the development of complica-

tions. Two who developed peritonsillar abscesses were started on penicillin therapy and were eliminated from this group. (The comparative results of penicillin and symptomatic treatment will be described elsewhere [4].)

"Treated" groups

The patients in these groups received the symptomatic therapy outlined above, including the obligatory period of bed rest. In addition, they received treatment with one of two penicillin regimens, which for convenience will be denoted Penicillin I and Penicillin II.

Penicillin I: This schedule entailed a single daily injection of 300,000 units of penicillin in aqueous solution for six to seven successive days. The rationale for this unusual and extravagant dosage will be presented later.

Penicillin II: Patients in this group (already mentioned) received penicillin in dosages comparable to usual experience; 20-50,000 units every three hours for from four to seven days. Most of the patients so treated (75 per cent) received penicillin for six or more days. The average period of therapy was 5.6 days, and the usual individual dose was 30,000 units. Selection of this regimen was predicated on the experience of Plummer and his co-workers, who found that clinical relapse occurred frequently in patients treated less than six days (5). According to Rammelkamp and Kirby (6), 20,000 units of penicillin "produces concentrations having maximal anti-streptococcal action for a period of more than two and one-half hours with partially inhibitory levels for another one and one-half to two hours." Thus, this dosage plan provided almost continuous levels effective against the hemolytic streptococcus.

DEFINITIVE DIAGNOSIS

It was realized from the outset that the diagnosis of streptococcal pharyngitis is difficult, and that there exist differences of opinion regarding the necessity of demonstrating a rise in the antistreptolysin titer for proof of diagnosis. The recent studies of Weinstein have indicated that in scarlet fever treated with long term (ten-day) penicillin therapy there is usually no antistreptolysin response (7). Thus, in an undoubted streptococcal infection, there is evidence that penicillin therapy may suppress antibody response. As it was realized that in the present study those cases which were treated with penicillin might show fewer instances of antibody response, rigid and exacting criteria for the final inclusion of patients in this series were established regardless of how clinically "typical" they had been on admission. The criteria were: (1) history and physical findings compatible with a diagnosis of streptococcal pharyngitis (as delineated earlier in this paper), (2) fever of greater than 100 degrees F. on the day of admission, (3) predominating growth of beta hemolytic streptococci on the initial throat culture, and (4) leukocytosis; i.e., a total leukocyte count greater than 10,000 cells per cu. mm.

The few cases which have been included which did not necessarily fulfill all four of these criteria, were cases in

which: (a) a significant antistreptolysin rise was demonstrated, or (b) the patient had a scarlatinal rash, or (c) peritonsillar abscess from which hemolytic streptococci were cultured existed at the time of admission.

The criteria were probably unduly severe. Plummer *et al.* noted that "an unexpected feature of (severe hemolytic streptococcal pharyngitis) was the slight increase or absence of increase in the total white blood cell count (5)." Rantz and his associates state that leukocytosis of "diagnostic value" is seen in only 49.1 per cent of patients (3) and that fever may be absent in patients with streptococcal pharyngitis (8). It seemed desirable to include only indisputable cases, however, in a study in which an important criterion of diagnosis was often lacking.

The application of the criteria plus the loss of patients by transfer and incomplete laboratory data reduced the series from 184 to 127 patients. The antistreptolysin rise in the "untreated" group (see Figure 1), however, affirms the accuracy of the preliminary diagnoses in a high proportion of instances.

RESULTS

The antistreptolysin response in patients receiving no specific treatment

In the group receiving no specific treatment, which included 51 patients, 84.3 per cent demonstrated a significant rise in antistreptolysin titer (Figure 1). This incidence is comparable to the value of other observers (9, 10). The average amplitude of the antistreptolysin rise was 462

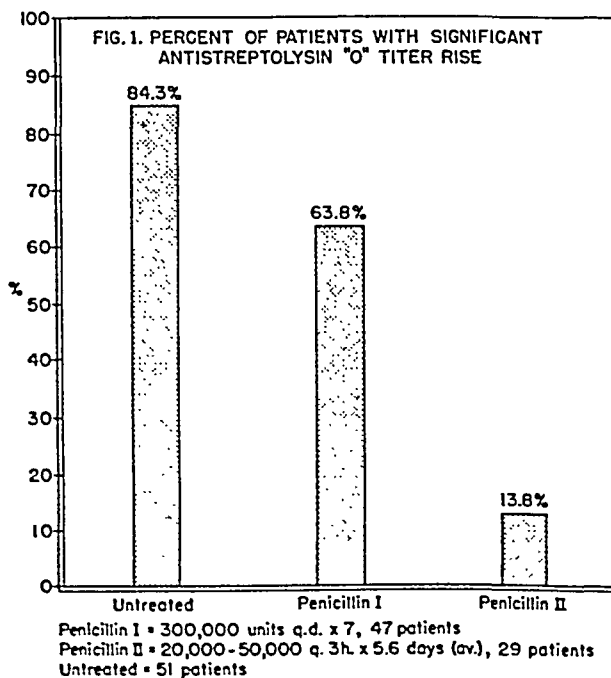


FIG. 1

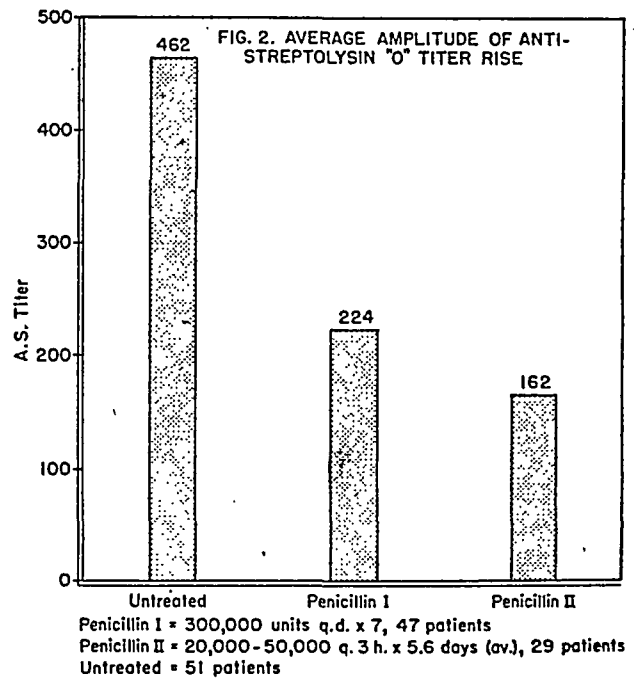


FIG. 2

units (Figure 2). The greatest rise (1150 units) is graphically shown in Figure 3.

The antistreptolysin response in patients receiving Penicillin Dosage I (large single daily dose)

63.8 per cent of 47 patients treated with Penicillin Dosage I exhibited a significant rise in antistreptolysin titer (Figure 1). This rise was of less amplitude than in the "untreated" group, averaging 224 units in patients with significant antistreptolysin responses (Figure 2). The extreme rise was 1050 units, comparable to that in the untreated group (Figure 3).

The antistreptolysin response in patients receiving Penicillin Dosage II (small frequent doses)

Only 13.8 per cent of the 29 patients treated with Penicillin Dosage II manifested a significant antistreptolysin rise (Figure 1). This rise was the smallest seen, and averaged 162 units in the four patients showing antibody response (Figure 2). The maximum rise exhibited by a patient in this group was only 300 units (Figure 3).

DISCUSSION

The results of the investigation indicate striking suppression of the incidence and degree of antibody formation by continuous penicillin treat-

ment and remarkably little inhibition of antibody response by intermittent penicillin therapy.

The apparent suppression of antibody formation in the penicillin-treated groups must be critically evaluated. It is essential to establish that patients in these groups had actually suffered streptococcal infection. The typical clinical appearance of the patients, and the exacting criteria used in their definitive diagnosis have been described. As some investigators believe that a rise in antistreptolysin titer is requisite proof of infection by the hemolytic streptococcus, reference is made to the high percentage rise in the "untreated" control group. In this series of alternately selected cases a similar rise is predictable in the penicillin-treated groups.

The percentages of patients exhibiting low concentrations of antistreptolysin were analyzed because Rantz has stated that the height of the initial level of antistreptolysin may affect the incidence or degree of rise in titer (11).

In the "untreated" group 68.3 per cent had initial levels of 100 units or less of antistreptolysin. In the group showing the least number of antistreptolysin responses (Penicillin II) the incidence of low initial levels was 65.5 per cent. Thus this factor may scarcely be construed as an important source of error.

Analysis of the untreated cases with high initial titers did not corroborate the finding of Rantz that the magnitude of rise was less in patients with high initial titers. The average rise in patients with initial levels of 200 or more units was 500 units, greater than the average rise in this group as a whole.

The apparent tendency of antimicrobial therapy to suppress the formation of antistreptolysin has been noted before. Rantz and his associates noted guardedly that an antistreptolysin response was observed less often in individuals receiving a long course of penicillin (80 hours), providing bacterial relapse did not occur, than with patients treated with short-course penicillin, sulfadiazine or salicylates (12). Earlier, Rantz, Kirby and Jacobs had presented "suggestive evidence that sulfonamide medication occasionally interfered with the development of agglutinins and antistreptolysin (13)." More recently, Weinstein and Tsao (7) have described suppression of the antistreptolysin formation in scarlet fever treated with a ten-day course of penicillin.

In analyzing the differing antibody responses with the two penicillin treatment schedules, it must be emphasized that although the 300,000 unit regimen (Penicillin I) provided a greater total dose and higher peak of serum concentrations, it effected detectable antibacterial levels for only a fraction of the 24-hour period. This is in contrast to the three-hourly dosage (Penicillin II), which maintained almost continuous detectable concentrations in the serum. It may be hypothesized that suppression of the antistreptolysin antibody by penicillin therapy is thus proportional to the duration of effective antibacterial action.

It is pertinent at this point to outline the rationale for the unusual penicillin dosage scheme denoted heretofore as Penicillin I.

Although the bulk of clinical and experimental evidence favors the use of multiple injections of penicillin in small dosage (6, 14), McDermott and his associates point out that "There is . . . reason to believe from clinical experience and from certain of the *in vitro* experiments that . . . prolonged maintenance of an effective level need not be absolutely continuous (15)." Tillet *et al.*, (16) have demonstrated that regimens utilizing widely spaced and small dosages of penicillin (10,000 units

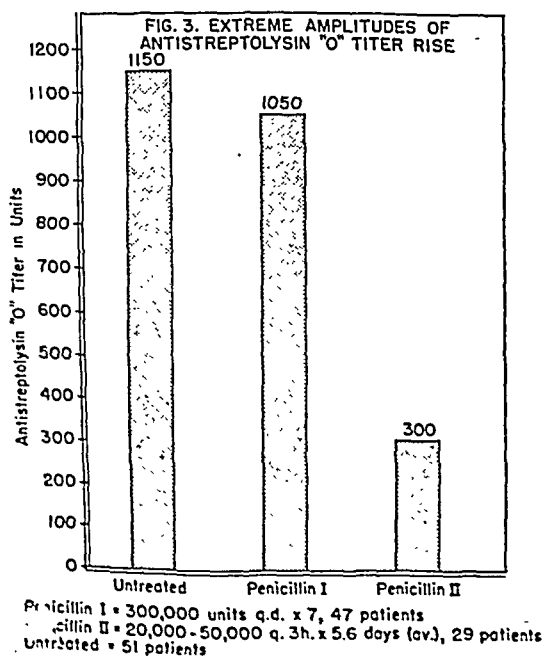


FIG. 3

intramuscularly four times daily) will effect clinical improvement or cure in pneumococcal pneumonia. In fact, 100,000 units administered intravenously every three hours for three doses effected a "cure" for 30 hours although followed by a sharp return of symptomatology (16). Tillett's experience has received recent confirmation by Tompsett and McDermott who have successfully treated acute bacterial infections with large doses of penicillin in aqueous solution injected only once or twice daily (17). The present authors have successfully treated a case of proved pneumococcal pneumonia with single daily doses of 300,000 units given for seven days.

In this epidemic of an acute bacterial disease, conservation of the time of the overburdened nursing personnel was important, and a regimen entailing infrequent injections was therefore desirable. Secondary but important considerations were the elimination of the discomfort attendant upon multiple injections; and the opportunity to acquire further clinical experience with this type of therapy.

Moreover, in studying the effects of penicillin on antibody formation it seemed important to distinguish between the effects of continuous and sporadic dosage. 300,000 units was selected as an amount which would maintain detectable concentrations (greater than .078 units per cc.) in the blood stream for five to seven hours, an appreciable fraction of the 24-hour period (18).

Significance of the antistreptolysin response

It is generally accepted that a rise in the titer of antistreptolysin is indicative of a recent infection with the beta hemolytic streptococcus. The specificity of this response was established by the classical work of Todd (1, 19), which has been extensively confirmed. Mote and Jones (9) showed that 78 per cent of cases of scarlet fever had an antistreptolysin rise in convalescence. More recently, Rantz, Boisvert, and Spink (10) have described a significant antistreptolysin response in 87.5 per cent of 342 patients with streptococcal sore throat. All three groups of workers (9, 10, 19) have also commented on the correlation of acute attacks of rheumatic fever and a rise in antistreptolysin titer. Similar rises have been noted in acute glomerulonephritis. Such evidence is basic to the present concept of rheumatic fever

and glomerulonephritis as non-suppurative sequelae of streptococcal infection.

The significance of the suppression of the antistreptolysin antibody is conjectural. Weinstein noted that of seven untreated cases which showed no increase in antistreptolysin, two suffered recurrent streptococcal pharyngitis, and one, recurrent scarlet fever; and of 29 penicillin-treated cases with no rise in antistreptolysin titer, two had recurrences of streptococci in the pharynx and concomitant fever.

Five relapses occurred in the present series of 127 cases, all followed for a minimum of 21 days (Table I). Three were in the group receiving

TABLE I
*Relapses**

	Number of relapses	Number with antistreptolysin rise
Untreated	0	0
Penicillin I (300,000 units q.d.)	2	1†
Penicillin II (20,000-50,000 units q. 3 h.)	3	0

* The follow-up observation period in all cases was at least 21 days. "Relapse" means return of the clinical, laboratory, and bacteriologic picture of streptococcal pharyngitis.

† The second antistreptolysin titer was drawn following the relapse.

"regular" penicillin therapy (Penicillin II), and it is perhaps significant that none of these had had a rise in antistreptolysin titer prior to relapse. Moreover, two of these patients received no antibacterial therapy on re-admission to the hospital and on this occasion developed antistreptolysin rises of more than 300 and 1100 units, respectively. This fortuitous experiment demonstrated beyond doubt the ability of these patients to form antistreptolysin. Rantz, Boisvert and Spink have cautioned that evidence of antibody suppression must be interpreted in view of the "great individual differences which exist between various human beings in their ability to react to the antigenic stimulus of infection . . . (12)."

The remaining two patients who showed clinical and bacteriological evidence of relapse had been treated with 300,000 units per day. Evaluation of these relapses in terms of antibody suppression is impossible as the final titers were not determined until after a second course of therapy.

It has been mentioned that attacks of acute rheumatic fever, as well as other late sequelae of hemolytic streptococcal infection, are frequently associated with a rise in antistreptolysin titer. In Weinstein's study (7) all the cases of rheumatic fever and of glomerulonephritis occurred in patients who developed a rise in antistreptolysin titer.

Analysis of cases in our study who developed late complications reveals data in agreement with this past experience. Five patients developed definite evidences of late sequelae. Two had undoubted rheumatic fever, one, severe arthralgia with elevated sedimentation rate, one had microscopic hematuria, and another prolonged fever of 19 days' duration (Table II). All five patients de-

TABLE II
Late systemic sequelae

	Number and description of cases	Number with antistreptolysin rise
Untreated	2 prolonged fever (1) microscopic hematuria (1)	2
Penicillin I (300,000 units q.d.)	3 rheumatic fever (2) ? rheumatic fever (1)	3
Penicillin II (20,000-50,000 units q. 3 h.)	0	0

veloped a rise in antistreptolysin titer. Three had been treated with intermittent high dosage penicillin while the two remaining cases were in the untreated group. No late sequelae have been observed to date in the group treated with sustained penicillin therapy.

SUMMARY

1. One-hundred-twenty-seven cases of hemolytic streptococcal pharyngitis were divided in rotation into three groups. One group served as an untreated control, one group was treated with large single daily doses of penicillin, and the third received virtually continuous penicillin therapy.

2. Antibody response, as measured by the antistreptolysin titers, was studied in all patients. The response varied in the three groups. In the untreated group the proportion of patients exhibiting a rise was comparable to that usually seen in streptococcal pharyngitis and scarlet fever. The

antistreptolysin response in the group treated with continuous penicillin therapy was of appreciably lower frequency and magnitude than that manifested by the untreated group, as previously described by Weinstein and Tsao. Patients who received intermittent penicillin treatment manifested an incidence and degree of antibody response intermediate between that of the untreated and continuously treated groups.

3. All patients who developed late sequelae had manifested rises in antistreptolysin titer prior to or coincident with the appearance of these sequelae.

4. No patient who relapsed had developed a rise in antistreptolysin titer during the initial illness.

CONCLUSIONS

1. Penicillin therapy suppressed the formation of antistreptolysin in hemolytic streptococcal pharyngitis.

2. The degree of suppression of antistreptolysin formation was proportional to the duration of the antibacterial action of penicillin maintained during the 24-hour period. The antibody response was suppressed more by the maintenance of continuous effective penicillin blood concentrations than by the maintenance of effective penicillin levels for a fraction (five to seven hours) of the 24-hour period.

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QUANTITATIVE ANTISTREPTOKINASE STUDIES IN PATIENTS INFECTED WITH GROUP A HEMOLYTIC STREPTOCOCCI: A COMPARISON WITH SERUM ANTISTREPTOLYSIN AND GAMMA GLOBULIN LEVELS WITH SPECIAL REFERENCE TO THE OCCURRENCE OF RHEUMATIC FEVER¹

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The phenomenon of liquefaction of human fibrin clots by broth cultures or culture filtrates of *beta* hemolytic streptococci was first described by Tillett and Garner in 1933 (1). The complex mechanism of this reaction, thought at first to be a direct fibrinolytic effect of a streptococcal enzyme, has been elucidated in recent years. Milstone (2) demonstrated that a substance present in plasma, termed "lytic factor," is required for dissolution of the clot; and as a result of the work of Christensen (3, 4), confirmed independently by Kaplan (5), it is now known that the "lytic factor" is a proteolytic enzyme normally present in the plasma as an inactive precursor. The role of the active streptococcal substance is that of an activator of the proteinase precursor, converting it into an active enzyme in a manner analogous to the conversion of trypsinogen to trypsin by enterokinase. The active serum proteinase is responsible for digestion of the fibrin clot. In view of the accumulated evidence regarding the nature of streptococcal fibrinolysis, Christensen and MacLeod (6) have proposed the term *streptokinase* to replace the term fibrinolysin originally applied to the streptococcal component of the system. They have further suggested the name *plasminogen* for the inactive form of the serum proteinase and *plasmin* for the active enzyme. This terminology has been adopted in the present report.

The activity of streptokinase derived from group A hemolytic streptococci in promoting the lysis of human fibrin clots is in sharp contrast to its minimal effect on clots of other animal species (1, 4, 7, 8). Although the basis for the apparent

human specificity remains somewhat uncertain, this property of streptokinase is unique and other products of streptococcal cells, such as streptolysin, erythrogenin, and streptococcal proteinase do not exhibit comparable specificity. Thus, streptokinase deserves special attention in the study of rheumatic fever, which is a sequela of streptococcal infection that appears to be limited to the human species.

Numerous immunological studies on streptokinase (fibrinolysin) have been carried out with human plasma (9, 10, 11, 12, 13) using the anti-fibrinolysin test described by Tillett and Garner (1), but because of the essentially qualitative nature of the test, it has not been possible to obtain definitive information comparable to that derived from the more precise quantitative titrations of other serum antibodies. Kaplan, in collaboration with the Commission on Acute Respiratory Diseases, published in 1946 (14) the first quantitative method for estimation of antibody directed against streptokinase. This method involves a neutralization test in which a constant, standardized amount of streptokinase is incubated with serial dilutions of the serum to be tested, following which an indicator system consisting of fibrinogen, plasminogen and thrombin is added. The end-point of the test is defined as the reciprocal of the highest dilution of serum which completely prevents lysis of the clot during a second period of incubation. The results of individual titrations proved to be sufficiently reproducible on repeated tests to establish the method as a useful quantitative procedure. The most serious objection that can be raised to the method is that it has not been possible up to the present time to standardize the plasminogen-streptokinase system in specific units so that antibody titers obtained in one laboratory can be compared directly with those of other lab-

¹ Assisted in part by a contract between The Rockefeller Institute for Medical Research and the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

oratories. In a given study, however, valuable data can be obtained by using a single lot of streptokinase and of human plasma Cohn Fraction I (containing both fibrinogen and plasminogen) for the entire series of tests.

In the present study, quantitative antistreptokinase determinations have been carried out on serial specimens of serum obtained from patients during an epidemic of scarlet fever. The primary aim of the investigation was to determine whether or not significant differences in the pattern of antistreptokinase response exist between patients who developed rheumatic fever subsequent to the attack of scarlet fever and those who did not. Antistreptolysin O titers and the gamma globulin levels of the same sera are presented for comparison.

MATERIALS AND METHODS

Buffered saline solution: 0.05M veronal buffer, pH 7.5, diluted to 0.01M with 0.85 per cent NaCl solution was used throughout in diluting the various reagents. This solution is hereafter referred to as buffered saline solution.

Fibrinogen and plasminogen: A lyophilized preparation of Fraction I (Cohn) of human plasma² served as the source of both fibrinogen and plasminogen. A 0.6 per cent solution in buffered saline was used both in standardizing the streptokinase preparation and in the quantitative antibody determinations. Solutions of this concentration formed a firm clot promptly upon the addition of thrombin.

Thrombin: Topical thrombin,³ of bovine origin, supplied in ampoules containing 5000 units, was dissolved in 5 cc. of physiological saline solution and further diluted 1:10 in buffered saline solution.

Streptokinase: A single lot of streptokinase was used throughout these experiments. A strain of group A hemolytic streptococcus, H105,⁴ was grown overnight in neopeptone dialysate broth (15). The next morning sterile 50 per cent glucose solution (40 cc. per liter of culture) and phenol red (10 mg. per liter of culture) were added to the culture and incubation continued another five hours during which time the pH of the culture was maintained at or slightly above 7.0 by the frequent addition of 5N NaOH. This procedure resulted in a tenfold increase of streptokinase activity. The culture was centrifuged and the supernate filtered through a Coors No. 3 filter. Approximately 300 cc. of non-dialyzed neopeptone broth had previously been passed through this filter, because it was found that a filter not so treated quantitatively absorbs streptokinase out of

dialyzed neopeptone broth. One-cc. amounts of the sterile filtrate were placed in small tubes and stored in a CO₂ chest at -70° C.

Standardization of streptokinase: The procedures used for the standardization of streptokinase and for the quantitative determination of antistreptokinase are similar to, but not identical with, those described by Kaplan (14). In each of a series of tubes were placed the following: 1.0 cc. of varying dilutions of streptokinase, 1.0 cc. of 0.6 per cent Fraction I solution, and 0.2 cc. of the thrombin solution. The tubes were immediately shaken and, when clotting had occurred, were incubated in a water bath at 37° C. for 30 minutes. The highest dilution of streptokinase which just effected complete dissolution of the fibrin clot was used in the determination of antistreptokinase.

Determination of antistreptokinase in serum: Twofold dilutions of serum were used, beginning with a dilution of 1:10. To 0.5 cc. of the serum dilutions was added 0.5 cc. of streptokinase in the dilution determined by the method outlined above. These tubes were mixed by shaking and then incubated in a water bath at 37° C. for 30 minutes to allow for antigen-antibody combination. According to Kaplan (16) this is 95-98 per cent complete in 30 minutes. The tubes were then placed in an ice-water bath to prevent as far as possible any enzymatic activity during the time required for the addition of the indicator system. To each tube was added 1.0 cc. of the 0.6 per cent Fraction I solution (containing plasminogen and fibrinogen). There was then added 0.2 cc. of the thrombin solution and the tubes shaken immediately to insure the formation of a uniform clot. The tubes were re-incubated at 37° C. for 60 minutes. The criteria for reading the rest are those described by Kaplan. The end-point was taken as the reciprocal of the serum dilution which completely prevented lysis of the clot as determined by the failure of the clot to slide when the tube was gently tapped in the inverted position. As a control on the activity of the streptokinase preparation, a tube in which the serum dilution was replaced by buffered saline solution was included in each series of dilutions. Complete lysis was expected in this tube. The largest number of sera which could be conveniently tested at one time was found to be six. An indication of the reproducibility of the test is given by the results of 50 repeat titrations. The end-points were identical in 38, and varied by only one tube in the remaining 12. Therefore, a rise in antibody titer of two or more tubes was considered to be significant.

Antistreptolysin O: The method used for the determination of antistreptolysin O was that described by Todd as modified by Hodge and Swift (17). A two-tube rise in titer was considered significant. It should be noted that the dilution increments in the determination of antistreptolysin O differ from those used in the antistreptokinase test.

Gamma globulin: The gamma globulin levels were determined by the turbidimetric method described by Kunkel (18). The turbidity readings were converted

² We are indebted to Sharp and Dohme, Inc., for a generous supply of Fraction I.

³ Manufactured by Parke, Davis and Co.

⁴ Strain H105 was obtained from Dr. W. S. Tillett and was designated Co in his laboratory (1).

into values expressed in gms. per cent on the basis of standardization of the turbidimetric readings by electrophoretic determination of gamma globulin.

Titration of the streptokinase produced by individual strains of group A hemolytic streptococci: The procedure used is similar to that reported by the Commission on Acute Respiratory Diseases (19). A lyophilized culture of the organism to be tested was inoculated into 5.0 cc. of Todd-Hewitt broth containing 0.1 cc. of defibrinated rabbit blood. After incubation for 22 hours, 0.05 cc. of the culture was transferred to 5.0 cc. of Todd-Hewitt broth and incubated at 37° C. for 18 to 20 hours. After this period of growth, two drops of 0.01 per cent phenol red solution were added to the culture and the pH was adjusted to pH 7.0-7.5 with 1N NaOH. The culture was then centrifuged and the supernate tested undiluted and in dilutions of 1:5, 1:10, 1:20, and 1:30. The

test system consisted of 1.0 cc. of the culture supernate diluted in buffered saline, 1.0 cc. of the 0.6 per cent Fraction I solution, and 0.2 cc. of the thrombin solution. The tubes were incubated in a water bath at 37° C. for 60 minutes, and the end-point was taken as that dilution of culture supernate which produced complete lysis of the fibrin clot. In order to avoid the possible effect of variations in the culture media on streptokinase production, a single lot of broth was used throughout.

Case material: The case material employed in this study was derived from an epidemic of scarlet fever in young adult males at the Great Lakes Naval Training Station, and all patients were admitted between February 26, 1946, and May 2, 1946. With the cooperation of the U. S. Naval Medical Research Unit No. 4, throat cultures and specimens of serum were obtained from these patients on admission to the hospital and at weekly

TABLE I
Detailed bacteriological and antibody data on 90 cases of group A hemolytic streptococcal infection

Group	Case	Streptococcus type*					Antistreptokinase† titer					Antistreptolysin O‡ titer					Gamma globulin grams per cent				
		0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.
I	49	30	30	30	30	30	40	40	40	80		250	300	300	300		0.72	0.86	0.86	0.89	
	123	30	30	30	30	30	40	40	80	160	160	150	150	200	200	150	0.83	1.05	1.05	1.05	0.98
	192	30	30	30	30	30	40	80	320	320		75	100	150	150		0.77	0.72	0.81	0.92	
	198	30	30	30	30	30	10	10	320	80		300	250	200	600		1.17	1.22	1.14	1.50	
	279	30	30	30	30	30	80	80	160	320	320	300	250	250	250	250	1.14	0.98	1.08	1.22	1.14
	290	30	30	30	30	—	40	80	160	160		150	400	300	250		0.81	1.17	1.17	1.14	1.14
	304	30	30	30	30	—	40	80	80	80	80	100	150	100	100	100	0.72	0.83	0.89	0.81	0.81
	11	30	NC	—	—	—	40	80	80	80		50	100	150	150		0.72	0.75	0.81	0.81	
	324	30	30	30	NC	—	160	160	640	640		150	200	250	250		0.77	0.77	0.81	0.89	
	63	19	19	19	19	19	10	20	40	40	40	150	150	200	250	200	0.83	0.92	0.92	0.98	0.89
	108	19	19	NC	19	19	0	40	160	160	160	0	100	200	200	100	0.89	0.89	0.92	0.92	0.98
	377	19	19	19	19	19	40	40	160	320		75	100	150	200		0.70	0.72	0.83	0.89	
	295	19	19	19	NC	19	20	40	40	40		50	100	100	100		0.64	1.17	0.92	0.92	
	316	19	19	19	19	19	160	320	640	640		75	100	150	100		0.77	0.95	0.75	0.86	
	131	17	17	17	17	17	20	320	320	320		25	250	200	250		0.62	0.72	0.77	0.98	
	288	3	3	3	—	—	40	40	40	40		150	250	400	500	100	0.83	0.98	1.00	1.14	0.89
	310	1	1	1	1	1	160	160	160	160		150	150	200	250		0.81	0.95	1.00	0.95	
	Average						55	99	216	214	140	129	184	213	241	150	0.81	0.91	0.93	0.99	0.95
II	55	17	30	30	19	NC	40	160	640	640		25	25	50	50		0.72	0.83	0.89	1.05	
	97	30	30	30	17	—	160	160	640	1280	640	0	100	150	250	200	0.95	1.08	1.38	1.55	1.38
	99	30	30	1	1	1	0	40	80	80		0	0	25	25		0.72	0.81	0.95	0.95	
	58	30	30	17	17	17	160	320	320	320		150	200	150	150		0.92	1.00	1.08	1.14	
	125	17	17	3	NC	30	20	80	320	80	320	75	250	500		800	0.77	0.89	1.00		1.50
	158	30	30	—	1	1	40	40	80	80		200	250	300	250	250	0.70	0.81	0.77	0.77	0.81
	222	30	19	1	1	1	80	80	320	320		150	200	400	250		0.83	0.83	1.08	1.22	
	235	3	3	30	30	30	40	80	80	80		100	150	200	250	300	0.81	0.89	1.05	1.05	1.14
	265	19	17	17	17	17	160	160	320	320		300	500	500	500		0.77	0.92	1.08	1.08	
	266	17	19	—	—	—	40	160	160	160	160	300	300	300	300	300	1.00	1.14	1.38	1.22	1.22
	208	17	17	1	1	1	80	320	640	320		250	250	300	250	300	1.00	1.22	1.44	1.41	
	14	17	NC	17	19	1	0	20	160	80	80	50	75	500	600	600	0.72	0.92	1.31	1.28	1.08
	Average						68	135	313	334	240	141	191	281	261	408	0.83	0.95	1.12	1.17	1.20
III	41	30	—	—	—	—	80	80	80	80		100	200	200	250		1.05	1.38	1.17	1.33	
	280	30	—	—	—	—	80	80	80	80		400	400	400	400		1.08	1.28	1.17	1.22	
	281	30	—	—	—	—	20	20	20	20	20	100	200	150	150	150	0.72	0.89	1.03	1.00	0.89
	94	19	—	—	—	—	20	20	20	20	20	25	25	25	25	25	0.72	0.77	0.72	0.86	0.77
	191	19	—	—	—	—	40	80	80	80		200	300	300	300		0.89	0.98	1.14	1.00	
	299	19	—	—	—	—	80	160	160	160		100	150	150	150		0.77	1.17	1.14	1.28	
	323	19	—	—	—	—	40	40	40	40		250	300	250	200		0.83	1.05	1.00	1.05	
	151	19	—	—	—	—	20	20	20	20	10	50	75	75	150		0.77	0.89	0.92	1.17	
	171	19	—	—	—	—	20	20	20	20	10	100	100	150	200		0.89	0.89	1.00	1.17	
	201	17	—	—	—	—	80	80	160	160		50	50	50	50		0.77	1.00	0.95	1.00	
	202	17	—	—	—	—	40	40	40	40		150	150	150	150		0.83	1.05	1.11	1.11	
	286	17	—	—	—	—	40	40	40	40	40	150	150	150	200	200	0.72	0.77	0.77	0.86	0.89
	261	17	—	—	—	—	20	20	40	40	20	75	200	150	150	150	0.77	1.22	1.03	1.05	1.05
	275	17	—	—	—	—	80	160	160	160		100	250	150	150		0.70	0.89	0.81	0.81	
	292	3	—	—	—	—	80	80	80	80	80	100	100	150	150	150	0.89	1.00	1.11	1.05	1.17
	258	3	—	—	—	—	40	20	20	20	40	50	100	100	100	100	0.67	0.67	0.77	0.77	0.83
	184	1	—	—	—	—	40	40	40	40	80	150	100	100	100	100	0.67	0.77	0.83	0.77	0.77
	Average						48	59	66	63	43	126	168	162	169	125	0.81	0.99	0.99	1.02	0.92

TABLE I—Continued

Group	Case	Streptococcus type*					Antistreptokinase† titer					Antistreptolysin O† titer					Gamma globulin grams per cent				
		0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.
IV	283	19	—	30	30	—	160	320	320	640	640	50	25	50	50	50	0.67	0.67	0.75	0.75	0.77
	289	17	17	—	17	17	20	80	640	640	640	50	200	1200	1200	1000	0.89	0.89	1.38	1.28	1.17
	300	17	—	17	—	—	20	40	40	80	—	100	100	150	150	—	0.67	0.67	0.72	0.67	—
	180	30	—	19	19	19	80	80	80	80	640	25	25	50	100	100	0.67	0.62	0.77	0.62	0.98
	309	30	—	—	19	19	160	320	320	320	320	100	100	150	150	200	0.77	0.83	0.77	0.75	0.75
	196	30	—	30	19	19	40	80	80	1280	2560	50	100	100	150	200	0.67	0.72	0.67	0.98	1.05
	334	19	—	30	—	—	80	80	160	160	640	150	150	250	250	250	0.89	1.17	1.11	1.17	1.17
	206	30	—	—	17	—	20	40	40	40	80	200	150	300	200	300	0.95	0.98	1.11	1.22	1.17
	267	30	—	30	—	—	80	80	80	80	—	250	250	250	250	—	0.75	0.89	0.95	0.98	—
	269	17	—	—	17	17	160	320	160	160	160	100	200	100	100	150	0.72	0.81	0.89	0.81	0.83
	249	30	—	3	—	—	320	640	640	640	640	250	250	300	300	400	0.62	0.67	0.67	0.72	0.77
	379	17	17	17	17	—	20	40	80	80	—	100	200	400	400	—	1.25	0.95	1.00	1.00	—
	54	17	17	17	17	—	40	80	160	160	—	100	150	150	200	—	0.62	0.59	0.72	0.75	—
	56	17	17	17	17	17	40	40	40	80	160	100	100	100	150	250	0.62	0.75	0.75	0.83	0.81
	68	30	30	17	NC	17	160	40	160	640	640	50	100	100	100	150	0.77	0.77	1.00	1.22	1.28
	71	19	19	19	1	—	0	0	320	320	—	25	25	150	200	—	0.81	0.95	1.11	1.11	—
	73	30	17	1	1	—	40	40	80	160	160	100	100	150	250	300	0.95	1.05	1.22	1.25	1.11
	126	19	17	17	—	—	320	320	320	—	—	640	200	250	300	300	0.75	0.75	0.67	—	0.95
	133	30	17	17	17	17	10	10	40	40	40	25	50	100	150	150	0.81	0.81	0.89	1.17	1.05
	305	30	—	19	—	—	160	320	160	160	160	200	300	300	300	400	0.75	0.81	0.81	0.83	0.83
	209	19	—	17	17	—	80	80	160	2560	—	100	100	100	500	—	0.77	0.81	0.83	1.44	—
	Average						96	145	194	416	541	111	139	226	257	286	0.77	0.82	0.89	0.98	0.98
V	13	30	—	—	—	1	80	80	320	1280	2560	150	250	300	300	300	1.02	1.28	1.44	2.48	2.64
	16	19	19	19	1	3	20	20	160	320	320	100	100	100	200	200	0.89	1.05	1.22	1.28	1.33
	23	17	—	17	17	17	40	160	640	640	640	150	250	300	700	300	0.95	1.02	1.38	1.33	1.28
	30	3	—	—	19	19	40	320	80	80	80	150	400	2000	3000	2000	0.95	1.11	1.67	1.38	1.55
	51	19	19	17	17	17	40	40	80	320	640	300	300	500	600	600	1.05	0.95	1.38	1.72	1.55
	52	17	17	17	3	3	160	160	320	640	640	100	200	250	400	1600	0.81	0.89	1.05	1.38	1.72
	65	19	19	19	—	—	20	20	160	640	640	250	250	300	700	500	0.89	1.05	1.38	1.83	1.67
	88	17	17	—	17	17	20	10	80	80	80	25	25	300	400	400	0.92	0.98	1.67	1.96	1.55
	92	1	1	30	17	—	40	80	160	160	640	100	150	300	500	1000	0.72	0.95	1.17	1.44	1.97
	120	30	30	30	—	—	80	80	320	640	1280	150	200	200	200	250	0.98	1.00	1.17	1.20	1.88
	234	19	19	—	—	—	80	160	320	640	640	1200	1600	1400	1200	1400	1.17	1.28	1.22	1.55	1.44
	247	—	—	17	17	17	640	640	640	1280	1280	150	200	200	200	200	0.92	1.08	0.95	1.08	1.08
	251	—	30	30	—	—	0	0	80	640	1280	200	200	200	500	700	0.81	0.81	0.95	1.83	1.88
	61	30	30	30	3	3	320	320	2560	2560	2560	100	150	250	300	400	0.67	0.75	1.00	1.00	1.05
	42	NT	NT	NT	NT	NT	40	80	80	80	80	100	150	200	150	150	0.77	0.77	0.95	1.00	1.17
	45	17	17	—	—	30	40	80	160	20	160	100	100	150	100	250	0.77	0.83	1.11	0.98	1.00
	181	30	—	—	17	17	40	160	320	320	320	200	600	1400	2000	2000	0.70	0.75	1.00	1.17	1.14
	175	19	—	17	17	—	0	0	10	40	80	250	250	250	250	250	1.05	1.22	1.14	1.14	1.22
	164	17	17	17	—	—	40	320	320	320	320	300	700	800	600	700	1.33	1.99	2.21	2.21	2.54
	173	30	3	30	17	—	160	160	320	320	640	100	150	150	250	250	1.17	1.33	1.44	1.28	1.17
	217	17	—	17	—	17	40	160	320	640	1280	250	300	300	500	800	0.89	1.05	1.00	1.17	1.55
	337	—	—	—	19	—	20	20	40	80	160	200	200	200	200	250	0.81	0.95	0.95	1.11	1.11
	44	30	17	17	—	30	160	160	160	320	640	250	200	500	600	700	1.05	1.44	1.38	1.33	1.50
	Average						92	140	332	524	737	212	301	459	602	661	0.92	1.06	1.25	1.43	1.52

* NC No culture obtained.

— No hemolytic streptococci.

NT Group A, no type.

† Antistreptokinase titers of less than 10, and antistreptolysin O titers of less than 25 are listed as 0.

intervals thereafter. The strains of streptococci isolated from the throat cultures were classified serologically in our laboratory by the precipitin technique of Swift, Wilson and Lancefield (20), and preserved for subsequent study by freezing and drying. The sera were stored at 4° C. Patients developing rheumatic fever were transferred to the Naval Hospital at Dublin, Georgia, and late specimens of serum were obtained from these individuals. Summaries of the clinical records of these patients were provided by the medical officers of Naval Research Unit No. 4.⁵

⁵ The authors are indebted to Lt. Comdr. John R. Seal, MC, USN, and the personnel of the U. S. Naval Medical Research Unit No. 4 for collection of sera and cultures and for supplying the clinical data on the patients. Dr. Robert F. Watson and Dr. Rebecca C. Lancefield represented the Hospital of the Rockefeller Institute for Medical Research in setting up this cooperative project.

As indicated by serological studies of the streptococcal strains isolated, three types of group A hemolytic streptococci (Types 17, 19, and 30) accounted for the great majority of the infections, but at least three other types (Types 1, 3, and one or more non-typeable strains) were involved in the epidemic. Individual isolation of the patients was not feasible, and the presence in the same ward of individuals infected with different types of streptococci resulted in a high incidence of cross infection. This occurrence of cross infection, together with other variables such as treatment and response to treatment, have been taken into consideration in the grouping of the case material chosen for serological studies. Cases were excluded from the study if clinical and bacteriological data were incomplete. Out of a total of 380 cases studied in the epidemic, detailed antibody studies were carried out on 90, grouped according to the following scheme:

Untreated, uncomplicated scarlet fever:

Group I: 17 patients from whom only a single type of group A hemolytic streptococcus was isolated. Although the failure to isolate more than one type of streptococcus from the serial throat cultures does not entirely eliminate the possibility of cross infections in these patients, it reduces the likelihood that clinically significant cross infections occurred.

Group II: 12 patients from whom more than one type of group A hemolytic streptococcus was isolated during the first two weeks of illness.

Penicillin-treated scarlet fever:

Group III: 17 patients from whom group A hemolytic streptococci could not be isolated after the initiation of treatment. Thus, from the bacteriological point of view, this group comprises those patients successfully treated with penicillin.

Group IV: 21 patients who showed persistence of, or recurrence of, a positive culture for group A hemolytic streptococcus of the same or different type.

Scarlet fever followed by rheumatic fever:

Group V: 23 cases. This group is not large enough to justify subdivision for purposes of analysis into sub-groups according to occurrence of cross infection and presence or absence of penicillin therapy. A majority of the patients were treated with penicillin, but none fell into the group of "successfully" treated cases comparable to Group III in which organisms disappeared permanently from the throat after the initiation of therapy. The selection of these rheumatic fever patients was of necessity based on rather rigid criteria, similar to those set down by other investigators. Unequivocal evidence of at least three of the following clinical or laboratory manifestations of rheumatic fever was required: arthritis or arthralgia; fever (subsequent to the initial streptococcal infection); elevated erythrocyte sedimentation rate; prolongation of the P-R interval (over 0.20 seconds); other electrocardiographic abnormalities; pericarditis; occurrence of valvular lesions as evidenced by characteristic murmurs. As a result of the use of these criteria, borderline and doubtful cases are not included. The objection that only the more severe cases of rheumatic fever have been accepted is perhaps valid, although in general these patients had relatively mild, monocyclic attacks, and few of them would be considered severe according to the usual clinical standards.

EXPERIMENTAL RESULTS

The results of antistreptokinase, antistreptolysin O, and gamma globulin determinations on the sera of the 90 patients are recorded in detail in Table I. Although certain facts are apparent from inspection of the table, the significant conclusions to be

drawn from the large mass of data are better illustrated by graphic treatment. The complete data are given for reference in conjunction with the figures which summarize the various aspects to be discussed.

In Figures 1-3, the arithmetic means of the three determinations are plotted against the week of disease. It will be seen at once from an inspection of these figures that, in general, the pattern of response within each group is similar in all three determinations. For example, the group of rheumatic fever patients (Group V) has consistently higher antibody titers and gamma globulin levels throughout the period studied than any of the other groups. The possible significance of this finding will be considered in detail below. In addition, there is a consistent difference between the degree of response of those untreated scarlet fever patients with single type infections (Group I) and those with multiple type or cross infections (Group II). The higher average response of the latter group suggests that the occurrence during infection of more than one type of streptococcus may augment the antibody response.

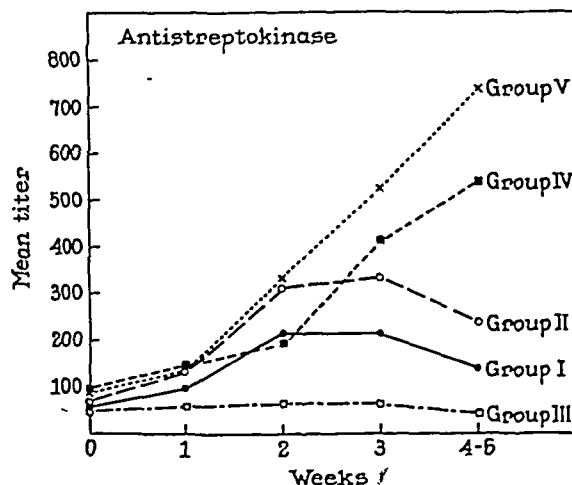


FIG. 1. MEAN ANTISTREPTOKINASE TITERS AT WEEKLY INTERVALS FROM TIME OF ADMISSION TO HOSPITAL

Group I: Uncomplicated scarlet fever; single type of streptococcus.

Group II: Uncomplicated scarlet fever; multiple types of streptococcus.

Group III: Penicillin-treated scarlet fever with disappearance of organism from throat.

Group IV: Penicillin-treated scarlet fever with persistence or reappearance of streptococci in throat culture.

Group V: Scarlet fever followed by rheumatic fever.

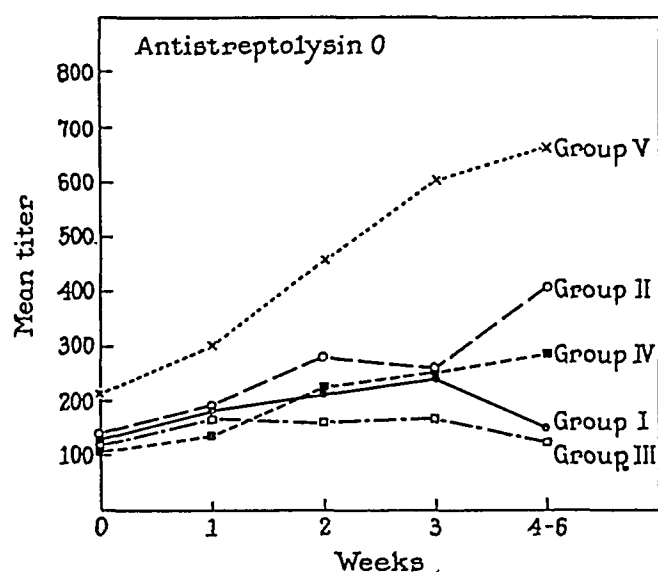


FIG. 2. MEAN ANTISTREPTOLYSIN TITERS AT WEEKLY INTERVALS FROM TIME OF ADMISSION TO HOSPITAL Groups as in Figure 1.

The failure of production of antibodies to streptokinase and streptolysin O in those patients "successfully" treated with penicillin (Group III) is apparent from inspection of Tables I and II, and of Figures 1 and 2. In no case was there a significant rise in antistreptokinase, while only eight of the 17 patients showed a significant, though minimal, response of antistreptolysin O. It should be remembered, however, that because of the differences in dilution increments used in the measurement of the two antibodies, a significant (or two-tube) rise in antistreptolysin O implies a smaller

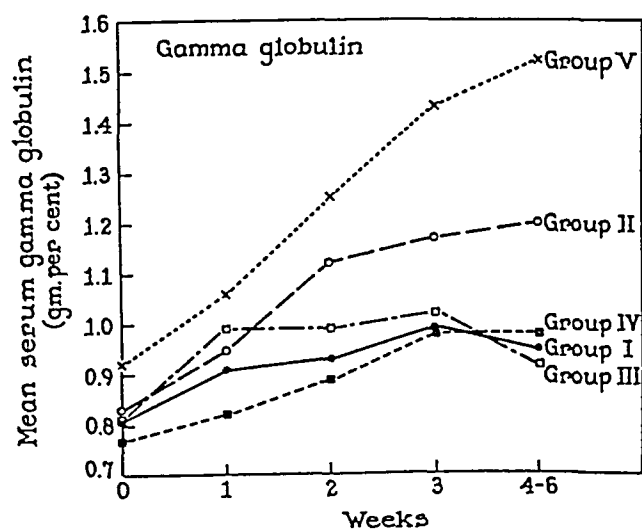


FIG. 3. MEAN SERUM GAMMA GLOBULIN LEVELS AT WEEKLY INTERVALS FROM TIME OF ADMISSION TO HOSPITAL

Groups as in Figure 1.

actual increase in antibody than in the case of antistreptokinase. Similar observations on the decreased antistreptolysin O response in patients with hemolytic streptococcal infections treated with large doses of penicillin have been reported by Rantz, Boisvert, and Spink (21), and by Weinstein and Tsao (22). In contrast to the flattened curves seen in Figures 1 and 2 for this group (Group III), the average gamma globulin levels (Figure 3) show a rise comparable to those of Groups I and IV. This suggests that the antibody response as a whole may not be greatly suppressed by the administration of penicillin. The two specific antibodies measured are directed against extracellular products elaborated by growing streptococcal cells, and may represent special cases with respect to the effect of penicillin on antibody production.

TABLE II

Percentage incidence of significant rises in antistreptokinase and antistreptolysin O titers

Group	No. of cases	Per cent showing significant rises in—			
		ASK	ASO	ASK and ASO	Either ASK or ASO
I	17	65	76	53	88
II	12	75	58	42	92
III	17	0	47	0	47
IV	21	71	76	62	90
V	23	87	83	74	96

The proportion of patients in each group showing significant rises in antistreptokinase and antistreptolysin O titers may be seen in Table II. With the exception of Group III, the incidence of significant responses of the two antibodies is similar within each group. The percentage difference in Group III is probably more apparent than real for the reason mentioned before. As demonstrated by the data given in the last two columns of Table II, patients with a significant antibody response to one antigen did not necessarily have a corresponding response to the other. The percentage of patients showing significant simultaneous rises in both antibodies is much smaller than the percentage showing a rise in either antistreptokinase or antistreptolysin O. No criterion for significance of an increase in gamma globulin has yet been rigidly defined, although with this

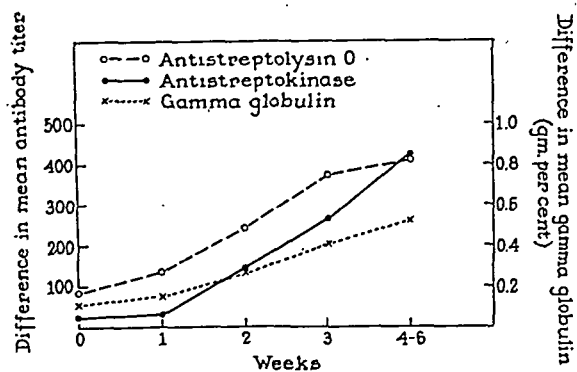


FIG. 4. DIFFERENCE IN ANTISTREPTOKINASE, ANTISTREPTOLYSIN AND GAMMA GLOBULIN LEVELS BETWEEN THE RHEUMATIC FEVER GROUP (GROUP V) AND THE NON-RHEUMATIC GROUPS (GROUPS I-IV)

method the normal range has been found to be about 0.67–1.0 gm. per cent (18).

The occurrence of consistently higher mean values for the two antibodies as well as for the gamma globulin levels in that group of patients with rheumatic fever (Group V) as compared with the other groups is worthy of special reference. Although the number of uncontrollable variables inherent in a study of this kind may cast some doubt on the validity of applying statistical methods, the results of statistical analysis of these data suggest that the observed differences are significant. For example, in the case of the mean value for the serum gamma globulin at three weeks after the onset of scarlet fever, the observed difference between the rheumatic fever patients and the entire non-rheumatic group is four times its standard error. According to statistical theory, the probability of this difference occurring by chance is 1 in 15,000.

There are additional considerations which appear to support the interpretation that the observed difference is significant. To provide graphic representation of the observations, the mean weekly value of each of the three determinations for the 67 scarlet fever patients in Groups I-IV have been subtracted from the corresponding mean values for the rheumatic fever patients (Group V) and the differences plotted in Figure 4. It will be seen that in the case of antistreptokinase and antistreptolysin O the absolute differences, as well as the rate of increase of the differences, are comparable. Although not directly comparable because they are expressed in different

units, the difference in the gamma globulin levels shows a rise similar to that of the antibodies. The mechanism and technical procedures involved in the three determinations vary widely. It is all the more striking, therefore, that the differences observed are of the same order in the case of each test.

The possibility must be considered that the arithmetic means for the various determinations may be unduly influenced by the occurrence of both very high and very low values with resultant exaggeration of minor differences. Consequently,

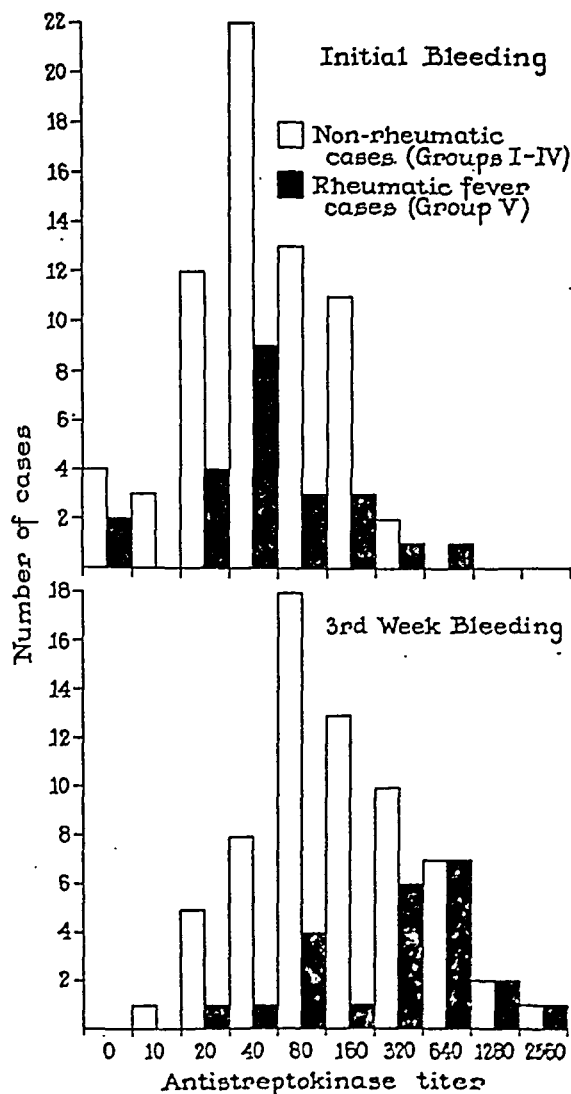


FIG. 5. COMPARISON OF DISTRIBUTION OF ANTISTREPTOKINASE TITERS OF RHEUMATIC (GROUP V) AND NON-RHEUMATIC (GROUPS I-IV) GROUPS AT TIME OF ADMISSION WITH SCARLET FEVER AND AFTER THREE WEEKS

frequency distributions were analysed for the three determinations, and are illustrated by the chart for antistreptokinase reproduced in Figure 5. At the time of the initial bleedings, the frequency distribution of the titers is remarkably similar for the two groups. By the end of 21 days, however, the two distributions have lost their similarity, and the one for the rheumatic fever group has shifted more to the right than has the one for the non-rheumatic fever group. Similar analyses of the antistreptolysin O and gamma globulin data yield comparable results. Thus this treatment of the data appears to support the significance of the differences between the rheumatic and non-rheumatic groups that was first suggested by the mean values of the various determinations.

In vitro production of streptokinase: Because previously published work (19) suggested that the antistreptokinase response of the patient was somewhat proportional to the quantity of streptokinase produced *in vitro* by the infecting organism, a number of group A hemolytic streptococci isolated from patients in this investigation were studied for their ability to produce streptokinase. Ninety-four assays were performed, using 62 different strains. Supernates of ten Type 1 and ten Type 3 streptococcal cultures were tested, and, without exception, all failed to lyse a standard clot even when tested undiluted. These two types were far less common during the epidemic than were the other types, so that untreated patients from whom only a Type 1 or a Type 3 streptococcus was isolated were few in number, and only two are represented in Table I. Neither has a significant antistreptokinase response. Forty-two group A hemolytic streptococci of Types 17, 19, and 30 were tested, and all produced measurable amounts of streptokinase. The supernates of two strains could be titered only to a dilution of 1:5; all the rest titered to dilutions of 1:10 or 1:20. There was no difference in ability to produce streptokinase between those organisms isolated from patients with rheumatic fever, and those from patients who did not develop rheumatic fever.

DISCUSSION

The results of the present studies provide no evidence that those patients who develop rheumatic fever following group A hemolytic strepto-

coccal infections have a pattern of antibody response to streptokinase that differs from the pattern of their response to other antigenic stimuli. On the contrary, the quantitative differences in antistreptokinase production which are observed in a comparison of the rheumatic and non-rheumatic patients are paralleled by comparable differences in the production of a second antibody, antistreptolysin O, and in the increase in serum gamma globulin. The data on gamma globulin suggest that the greater production of the two antibodies in those patients who developed rheumatic fever is a reflection of a general augmentation of antibody formation.

It is important to point out that in the present study the increased antibody production is made apparent only by the collective analysis of the two groups of cases, and no basis is provided for differentiating between individual patients. Thus, some of the patients with uncomplicated scarlet fever showed a greater rise in antibody titer than certain of the rheumatic fever patients, and a few of the latter have relatively insignificant increases.

Quantitative differences in production of antibody have not been emphasized in previous investigations of group A hemolytic streptococcal infections in which rheumatic and non-rheumatic patients have been compared (23, 24, 25, 26, 27). In a recent study, Rothbard, Watson, Swift, and Wilson (28) found that the average antistreptolysin O response, as indicated by the ratio between the maximal titer and initial titer, was greater in patients who developed rheumatic fever following a streptococcal infection than in those who had uncomplicated infections. However, they found in addition that a group of patients with purulent complications showed a much greater average response than either of the other two groups. The fact that the differences described in the present study are so readily apparent may be in some degree due to the homogeneity of the case material. These patients were selected from a single epidemic of scarlet fever occurring within a period of two months, and a relatively small number of streptococcal strains was involved. Furthermore, the patients represented an unusually uniform sample of the population, since all were males between the ages of 17 and 27; 82 (91 per cent) of the men were included in the age group of 17 to 20 years.

Accordingly, some of the uncontrollable variables commonly present in this type of study were eliminated, and it is reasonable to suppose that small differences of the sort described might become apparent under these conditions.

The possible significance of the apparent enhancement of antibody formation in the rheumatic subjects is difficult to assess. Conceivably it might mean that on the average these patients received a greater antigenic stimulus in the form of a more serious or extensive streptococcal infection, although there is nothing in the clinical histories to support this point of view. A second possible interpretation is that persons susceptible to rheumatic fever may in general respond to a given stimulus with greater production of antibody. It is well known, for example, that individual differences in degree of antibody formation occur among experimental animals injected with identical amounts of antigen. It is also worthy of note that even at the onset of the streptococcal infection, those patients who later developed rheumatic fever had, on the average, higher antibody titers and higher gamma globulin levels than did those who did not develop rheumatic fever. Regardless of the interpretation one wishes to put on the results, it seems premature and fruitless to attempt to reconcile them with any of the current theories concerning the pathogenesis of rheumatic fever.

SUMMARY AND CONCLUSIONS

1. A procedure for the quantitative determination of antistreptokinase has been employed to follow the antibody response of patients with scarlet fever, including those who developed rheumatic fever. Antistreptolysin O titers and gamma globulin levels on the same sera are presented for comparison.

2. There is suggestive evidence that the presence of two or more types of streptococci during an infection calls forth a greater antibody response than does the presence of only a single type.

3. Early and effective penicillin therapy which removed the infecting organism promptly from the nasopharynx either prevented entirely or greatly decreased the expected antibody response to streptokinase and streptolysin O. No effect was apparent on the total antibody response as measured by the serum gamma globulin.

4. Development of the rheumatic state is not accompanied by a pattern of antistreptokinase response that differs significantly from the pattern of the general immune response in the same state.

5. Of the patients included in this study, it would seem that on the average those who developed rheumatic fever as a sequela to a streptococcal infection exhibited a greater antibody response than those who did not develop rheumatic fever.

ACKNOWLEDGMENT

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THE EFFECT OF HEPARIN AND DICUMAROL ANTICOAGULANT THERAPY UPON THE ERYTHROCYTE SEDIMENTATION RATE

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Anticoagulant therapy has been employed in thromboembolic disease with increasing frequency during the past few years. There has been a striking lack of unanimity in the literature (1 to 15) as to the effect of heparin or dicumarol per se upon the erythrocyte sedimentation rate. In view of the fact that anticoagulants are frequently employed in disorders in which the sedimentation rate is of importance in diagnosis as well as in judging their progress, definitive information concerning the effect of anticoagulants on the erythrocyte sedimentation rate would be of considerable practical value.

The sedimentation rate of heparinized blood has been reported to be greater than that of citrated or oxalated blood (1, 2). This has been ascribed not to an accelerating effect of heparin but rather to a stabilizing and retarding influence exerted by the oxalate and citrate salts upon the red blood cell sedimentation (1 to 5). Conflicting observations have been reported (6, 7) as regards the effect upon the citrated sedimentation rate when heparin was added *in vitro* to blood specimens. It has been noted (5, 8) that heparin in unusually large concentrations resulted in an acceleration of the sedimentation rate. Furthermore, during venous clotting time determinations in a patient receiving heparin therapy, it is a frequent observation that the plasma and cells of the blood of a well heparinized patient will separate more rapidly than usual in the clotting time test tubes. This phenomenon has been remarked on (9, 10) and has given the impression that in a patient receiving heparin therapy the sedimentation rate is quite briskly increased.

Previous observations as to the effect of dicumarol on the sedimentation rate also are not in accord (11 to 15). It has been reported (11) that the erythrocyte sedimentation rate is not elevated by dicumarol therapy per se. However, in several later communications (12 to 14) it was stated that the sedimentation rate was increased by dicumarol to such an extent that its reliability and depend-

ability as a guide to healing was compromised. Recently one of these groups (15) has reversed its original stand (12) and stated that dicumarol probably has little, if any, effect on the sedimentation rate.

The present study was initiated to determine whether the sedimentation rate may still be accorded clinical significance in a patient who is receiving anticoagulant therapy.

METHODS

Venous clotting times were determined according to a modification of the Lee-White technique (16). Four chemically clean, 75 × 10 mm. test tubes, syringe and needle were rinsed with physiological saline solution. Following a cleanly negotiated venipuncture, 1 c.c. of blood was gently placed in each tube. Clotting was considered to have occurred when the blood did not flow when the tube was completely inverted and when there was no mixing of the red cell and plasma layers. Normal range was five to 12 minutes.

Prothrombin time determinations were performed on whole plasma, according to a modification (17) of Quick's technique (18) using 0.1 Molar sodium oxalate as the anticoagulant, commercially supplied rabbit lung as the source of thromboplastin, and 0.025 Molar calcium chloride. Normal prothrombin time in this laboratory with the technique employed was found to be 14 (± 2) seconds. The per cent of normal prothrombin activity was determined from the saline dilution logarithmic prothrombin activity curve constructed according to Quick (18). By this technique, 14 seconds equals 100 per cent normal prothrombin activity, 22 seconds equals 30 per cent of normal prothrombin activity and 45 seconds equals 10 per cent of normal prothrombin activity.

The erythrocyte sedimentation rate was determined by the Westergren method (19), using 0.5 c.c. of 3.5 per cent sodium citrate as the anticoagulant for 2.5 c.c. of venous blood. The sedimentation rate was determined immediately in glass tubes of standard bore (2.5-3.0 mm.) with the blood column at the constant height of 200 mm. and expressed as millimeters of fall in one hour. Normal values by this technique are considered to be less than 20 mm. in one hour.

Technique of anticoagulant administration. Dicumarol was administered orally in the usual dosage of 300 mgm. on the first day, 200 mgm. on the second day and subsequent dosage depending on the result of the daily prothrombin time. Heparin sodium was administered intravenously in amounts of 50 mgm. (1 c.c. = 10 mgm.).

RESULTS

The effects of the intravenous administration of 50 mgm. of heparin sodium were observed in ten subjects in whom the sedimentation rates had been static for a period of time before the start of the experiment. Sedimentation rates and venous clotting times were obtained immediately prior to the heparin injection, 30 minutes subsequent to the heparin injections and in some

TABLE I

The effect of the intravenous injection of 50 mgm. of heparin upon the erythrocyte sedimentation rate of ten subjects

Case	Determination	Time in minutes after heparin					
		Control	30	60	90	240	300
1	V. C. T.*	8	65		55	9	
	E. S. R.†	25	20		20	29	
2	V. C. T.	8	50			16	
	E. S. R.	4	1			2	2
3	V. C. T.	9	113				12
	E. S. R.	120	121				122
4	V. C. T.	10	90				8
	E. S. R.	2	3				2
5	V. C. T.	17	91				
	E. S. R.	8	8				
6	V. C. T.	18	143				
	E. S. R.	21	22				
7	V. C. T.	15	113				
	E. S. R.	10	10				
8	V. C. T.	14	107				
	E. S. R.	82	81				
9	V. C. T.	20	110				
	E. S. R.	92	112				
10	V. C. T.	20	220				
	E. S. R.	8	5				

* V. C. T. = Venous clotting time in minutes.

† E. S. R. = Erythrocyte sedimentation rate in millimeters per hour.

instances at later intervals. The results are presented in Table I. It was observed that in amounts of heparin which prolong the venous clotting time to between 50 and 220 minutes the sedimentation rate remains relatively constant and is not significantly increased or decreased.

The effect of dicumarol on the sedimentation rate was determined in ten individuals with static sedimentation rates. Sedimentation rates and pro-

thrombin times were determined prior to dicumarol administration and at intervals of one to three days during and subsequent to dicumarol therapy. The dicumarol was given as outlined in dosage sufficient to produce the degree of hypoprothrombinemia usually encountered during routine dicumarol anticoagulant therapy for thromboembolic disease. The results presented in Table II demonstrate that the sedimentation rate is not significantly altered and is remarkably constant during dicumarol administration.

It has been previously reported (20) that heparin and dicumarol are synergistic, in that the effect of heparin was enhanced by the concurrent administration of dicumarol. The effect of combined heparin and dicumarol administration upon the sedimentation rate was studied in five subjects, since it was felt that the concurrent administration of these anticoagulants possibly might be sufficient to alter the sedimentation rate. In these five individuals the sedimentation rate was not significantly altered by the preliminary administration of dicumarol at dosage levels sufficient to produce a marked hypoprothrombinemia. The injection of 50 mgm. of heparin sodium superimposed on the already established dicumarol prothrombin deficiency did not effect any significant change in the sedimentation rate, despite the production of marked changes in the venous clotting time.

SUMMARY

In the dosage employed clinically, heparin and dicumarol do not significantly alter the erythrocyte sedimentation rate.

The erythrocyte sedimentation rate of a patient receiving anticoagulant therapy can be considered to be as reliable a guide in the management of such an individual as it is in patients not receiving anticoagulants.

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SENSITIVITY OF SKELETAL MUSCLE TO INTRA-ARTERIAL ACETYLCHOLINE IN NORMAL AND MYASTHENIC MAN

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The weakness and fatigability of skeletal muscle in myasthenia gravis have been likened to similar phenomena which occur in curare poisoning. As the intensity of curarization increases, muscle groups weaken in a certain order. The same muscle groups are often affected in the same order as myasthenia gravis progresses. The mechanism of death by interference with respiration is often strikingly similar in the two conditions. These facts have suggested that myasthenia gravis may result from the presence in the body of a curare-like substance. In favor of this hypothesis could be cited (1) the great sensitivity of myasthenic patients toward curare (1), and (2) evidence for the production of this kind of substance by the muscles of myasthenic patients during exercise (2, 3).

One of the effects of curare is to diminish or abolish the stimulating action of acetylcholine upon the neuromuscular endplate. In experimental animals, curarization produces simultaneously a decrease in the amplitude of the muscular twitch on stimulation of the motor nerve and a decrease in the twitch-like response to acetylcholine (4). If, as seems likely, this antagonism to acetylcholine is the factor responsible for the neuromuscular block of curare poisoning, then the phrase "curare-like" action implies the diminution or abolition of the stimulating effect of acetylcholine in skeletal muscle. If the hypothesis is correct that the weakness of myasthenia gravis is due to the presence of a curare-like substance, the myasthenic muscle should be *less* sensitive than normal to stimulation by acetylcholine. Two groups of investigators (5 to 8) have compared the effects of relatively *large* doses of intra-arterially injected acetylcholine upon skeletal muscle in normal and myasthenic man. Both drew the conclusion that myasthenic muscles are *more* sensitive to acetylcholine than normal muscles. The aim of the observations reported below was to check this conclusion by determining

the response of normal and myasthenic muscles to threshold concentrations of acetylcholine.

The previous conclusions could not be confirmed; myasthenic muscles were not notably more or less sensitive to acetylcholine than normal muscles.

METHODS

The injections of acetylcholine¹ were made through a 24 or 25 gauge needle into the radial artery at the wrist or the brachial artery just above the tendon of the biceps muscle. The acetylcholine was injected within one to three seconds usually in a volume of 0.2 cc. Each subject received a number of injections (from two to six) at intervals of 30 to 60 seconds. The acetylcholine was used within an hour after it had been dissolved in sterile physiological saline solution.

The forearm was fixed to a frame clamped to a table edge in a position comfortable to the subject. When injections were made into the brachial artery, the slightly flexed fingers pulled against a gauze band attached via a pulley to a lever. When injections were made into the radial artery, the supine hand was held dorsiflexed by a padded bar and the opposing motion of the thumb was recorded by attaching a band held by adhesive tape to the metacarpophalangeal joint via a pulley to the writing lever. Rubber bands from the lever to a fixed point gave a degree of tension to the fingers or thumb such that slight voluntary movements were readily recorded, whereas strong voluntary contractions gave wide excursions of the writing point. The inscription was upon smoked paper.

In order to determine whether or not the doses of acetylcholine which were injected affected the transmission of nerve impulses across the neuromuscular junction, maximal stimulation of the appropriate motor nerves was sometimes carried out. The stimuli were condenser discharges (0.1 microfarads discharging through the subject). A broad, moist contact to the forearm or upper arm served as the anode, and a restricted contact through cotton wet with saline solution was the cathode. When injections were made into the brachial artery and the flexors of the fingers and wrist were arranged to record, the stimulating cathode was placed over the ulnar nerve at the elbow. As the voltage of the stimulus was increased, the twitches increased in amplitude to a maxi-

¹ Kindly supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey.

mum and then decreased somewhat. The voltage of the stimulus which made the largest twitch was used in subsequent observations. When injections were to be made into the radial artery and the opposing muscle of the thumb was arranged to record, the cathode was placed over the median nerve in the wrist. As the voltage of the stimulus was increased the twitches of this muscle quickly reached a maximal height and suffered no decline at higher voltages. In the subsequent observations, 1.5 times the smallest voltage producing maximal twitches was used.

The subjects were asked to make no voluntary motions of the arm or hand except on command, and to describe their experiences after the completion of the observations.

Since the five myasthenic subjects showed only moderate weakness, it was possible to omit Neostigmine Bromide medication for 12 to 20 hours before the injections were made. After a period of this duration without Neostigmine, the cholinesterase activity of the plasma, as measured by the method of Ammon (9), was between 80 and 100 per cent of normal values in three of these patients. One patient received a subcutaneous injection of 0.6 mgm. of Atropine Sulfate shortly before the intra-arterial injections of acetylcholine were performed. Each myasthenic subject gave evidence in both history and physical examination of slight to moderate weakness and fatigability of the muscles of the forearm and hand, which diminished after Neostigmine. Subjects E and H of Table I exhibited less weakness of these muscles than the other three subjects.

RESULTS

As in previous observations of the effects of intra-arterial acetylcholine (5 to 8, 10 to 12), no systemic effects of acetylcholine were noted. Except for the contractions of skeletal muscle to be noted below, the phenomena observed did not differ

from those noted by previous investigators. Each successful injection produced a marked flush and sweating of the area supplied by the artery into which the injection was made. With the smallest doses the subject reported a sensation of warmth or heat spreading peripherally over this area. With larger doses this sensation was intensified and accompanied by a sensation of tingling or pins-and-needles. As the doses increased the warmth or heat acquired a painful quality still referable mainly to the skin. Added to these were the sensations of muscular tension accompanying the contractions of skeletal muscle to be described below. With the largest doses this tended to be painful. All these sensory phenomena disappeared within 15 to 30 seconds after each injection. The flush, however, gradually disappeared in a period of about five minutes.

I. Myasthenic and normal subjects; brachial artery and flexors of the wrist and fingers

Acetylcholine chloride was injected in a volume of 0.2 cc. except in one myasthenic subject, in whom the doses were injected in a volume of 1.0 cc. In each subject, a series of ascending doses was injected. The smallest dose used (40 μ g.) produced no contractions of skeletal muscles (three subjects). The next greater dose (125 μ g.) produced small contractions in three out of five normal subjects but failed to do so in two myasthenic subjects. Doses of 300 and 420 μ g. of acetylcholine produced contractions in two other my-

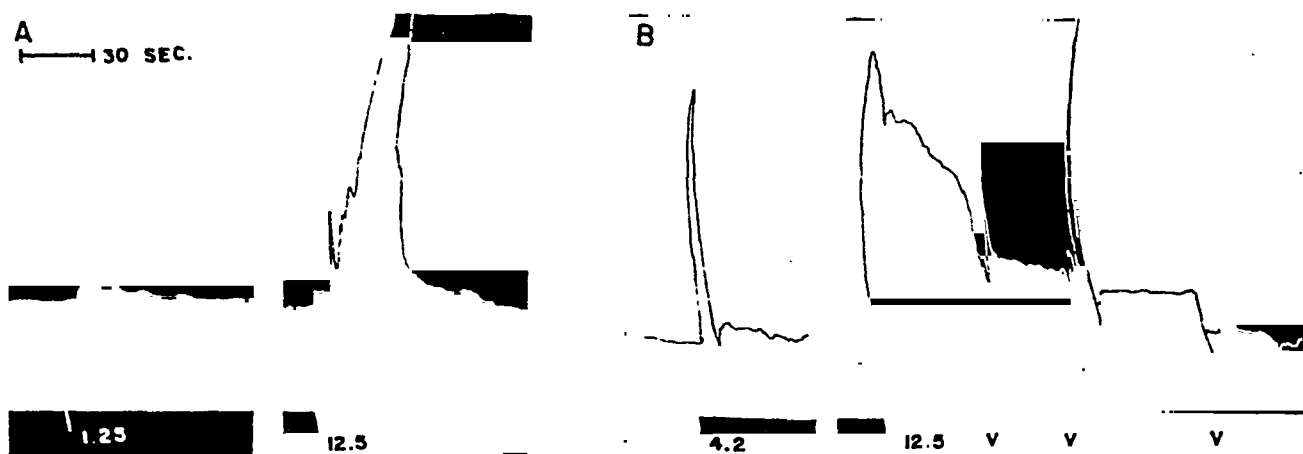


FIG. 1. CONTRACTIONS OF FLEXOR MUSCLES OF FINGERS AND WRIST PRODUCED BY RAPID INJECTION OF ACETYLCHOLINE INTO BRACHIAL ARTERY

The dose in milligrams is indicated below the signal line. A. Normal subject (CB of Table I). B. Myasthenic subject (MK of Table I). V indicates voluntary contraction or relaxation.

asthenic subjects. In a myasthenic subject 1.0 mgm. failed to produce contraction whereas 3 mgm. elicited a small contraction. In Table I, responses to the various doses injected in each of the ten subjects are listed in terms of the millimeters of excursion of the writing point of the myographic lever.

Figure 1 illustrates the contractions recorded in a normal (A) and a myasthenic (B) subject. The prolonged activity seen in the second tracing from the latter was also observed in normal subjects, and hence cannot be considered as characteristic of the myasthenic subjects. No qualitative or quantitative aspect of the contractions of the myasthenic subjects differed from those in the normal subjects.

Discussion

Previous investigators (5 to 8) alluded to an exquisite sensitivity of myasthenic muscles to acetylcholine as compared to normal. They interpreted this as indicating a compensatory sensitization of the muscles toward acetylcholine, resulting from the underlying cause of myasthenia gravis. An examination of the observations these investigators made, however, shows that no real basis existed for a statement about the sensitivity of normal and myasthenic subjects. Acetylcholine was injected into the brachial artery in doses of 10 to 50 mgm. (the largest dose of the present report was 12.5 mgm.). No attempt was made to find the smallest effective dose. In myasthenics these doses elicited strong contractions, whereas in normal subjects these large doses produced no contractions. The statements about sensitivity to acetylcholine were based upon the fact that these doses produced contraction in myasthenics but no contraction in normal subjects.

The observations reported here establish that when smaller doses of acetylcholine are injected intra-arterially in man, contractions of the skeletal muscles occur in both normal and myasthenic subjects. The aim of these observations was to determine whether or not there is a difference between the minimal dose of acetylcholine which stimulates the skeletal muscles in these two types of subjects. In the small groups studied, no significant difference either in threshold or in the character of the responses could be detected. Recent observations by Harvey, Lilienthal, and Talbot (13) confirm this conclusion.

Using much larger doses of acetylcholine (10 to 50 mgm.) the previous investigators (5 to 8) observed that strong contractions of the muscles of the hand and forearm occurred in myasthenic subjects, whereas no contraction occurred in normal subjects. This difference between normal and myasthenic subjects did not appear in the observations reported above, using doses from 0.04 to 12.5 mgm. of acetylcholine. It does not depend upon a difference of threshold of stimulation by acetylcholine. In a recent paper, Wilson and Stoner (14) reported that even with doses of 40 mgm. of acetylcholine no difference between normal and myasthenic subjects could be found.

Among the normal and myasthenic subjects, the thresholds to acetylcholine, as established by the procedure used, differed widely (see Table I).

TABLE I
Response by subjects in mm. excursion of myograms

Dose in mgm.	Normal					Myasthenic				
	GA	OK	M	O	CB	MK	D	H	E	AB
0.042	0		0	0						
0.125	0	9	3	6	0	0		0		
0.3									6	
0.42							10			
1.0							17		40	0
1.25		17	6	27	3			4	42	8
3.0										
4.2						60				
5.0									57	
12.5		9	11	100	75	72		25		

Three normal subjects responded to 0.125 mgm.; another had but a slight response to ten times this dose. The myasthenic thresholds were distributed over a similar range. It seemed desirable to investigate this variability further. Additional series of observations were therefore carried out in normal subjects, as described in the succeeding section.

II. Normal subjects: radial artery and opposing muscle of thumb

In five normal subjects, an ascending series of doses of acetylcholine chloride was injected into the radial artery while the motor fibers to the opposing muscle of the thumb were being stimulated maximally once each 4.2 seconds. The smallest dose (10 μ g.) never produced muscular contraction or tingling, the next dose (30 μ g.)

often produced a slight contraction of the opposer of the thumb accompanied by tingling sensations. In this series the succeeding doses (100 μ g., 300 μ g., and 1 mgm.) invariably produced contractions, usually of ascending amplitude and duration. In the series of observations to be reported in the next two paragraphs, some subjects had no response to 100 μ g., but all who received 200 μ g. exhibited muscular contractions.

Figure 2 illustrates responses to ascending doses of acetylcholine. The two tracings, A and B, are from two subjects. The upper tracing shows brief contractions with all except the largest dose. The lower tracing illustrates the more prolonged contractions obtained in some subjects. No significant change in the amplitude of the twitches elicited by nerve stimulation occurred as a result of these injections of acetylcholine.

In a second series of subjects the same dose of acetylcholine chloride (30 or 100 μ g.) was injected serially in different volumes of fluid (0.1, 0.2, 0.4, 1.0, and 0.1 cc. or double these volumes). Whereas usually some muscular contraction oc-

curred with one or another of these injections, the results were otherwise not predictable.

Hence a third series was undertaken in which the same dose of acetylcholine (100 or 200 μ g.) was injected repeatedly in a volume of 0.2 cc. In eight subjects, a muscular response occurred with one or more of these injections. The magnitude of the responses and the intensity of the sensory phenomena accompanying these responses, however, varied considerably from one to another injection in the same subject. The type and degree of variation are illustrated in Figure 3.

DISCUSSION

These further observations in normal subjects confirm the variability in threshold to acetylcholine reported for normal and myasthenic subjects in Section I. Some subjects responded to 30 μ g. and others needed 200 μ g. of acetylcholine. These observations further indicate that even in single normal subjects there is considerable variation in the responses to the same dose of acetylcholine injected in the same volume of

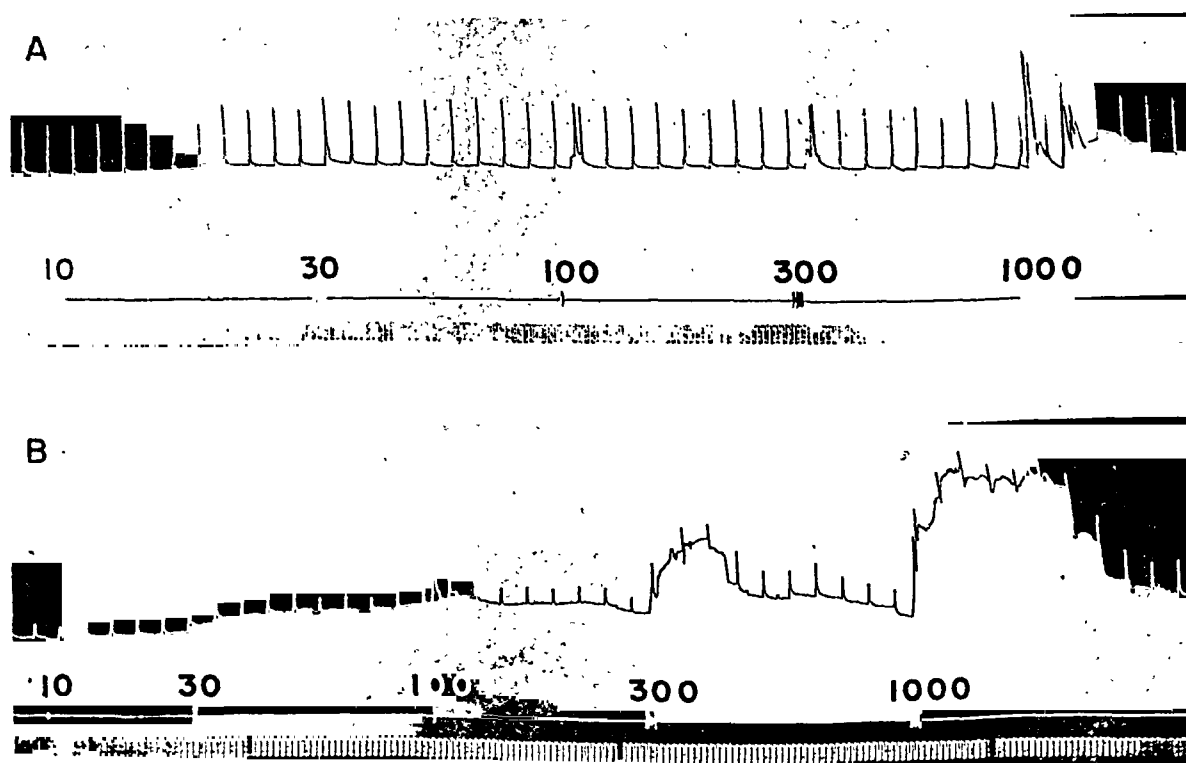


FIG. 2. CONTRACTIONS OF OPPOSING MUSCLE OF THE THUMB PRODUCED BY RAPID INJECTION OF ACETYLCHOLINE INTO RADIAL ARTERY AT WRIST

Throughout the period illustrated, there was maximal stimulation of the median nerve in the wrist every 4.2 sec. Doses are indicated in micrograms above the signal line. Bottom line: time in seconds and minutes. A and B: two normal subjects.

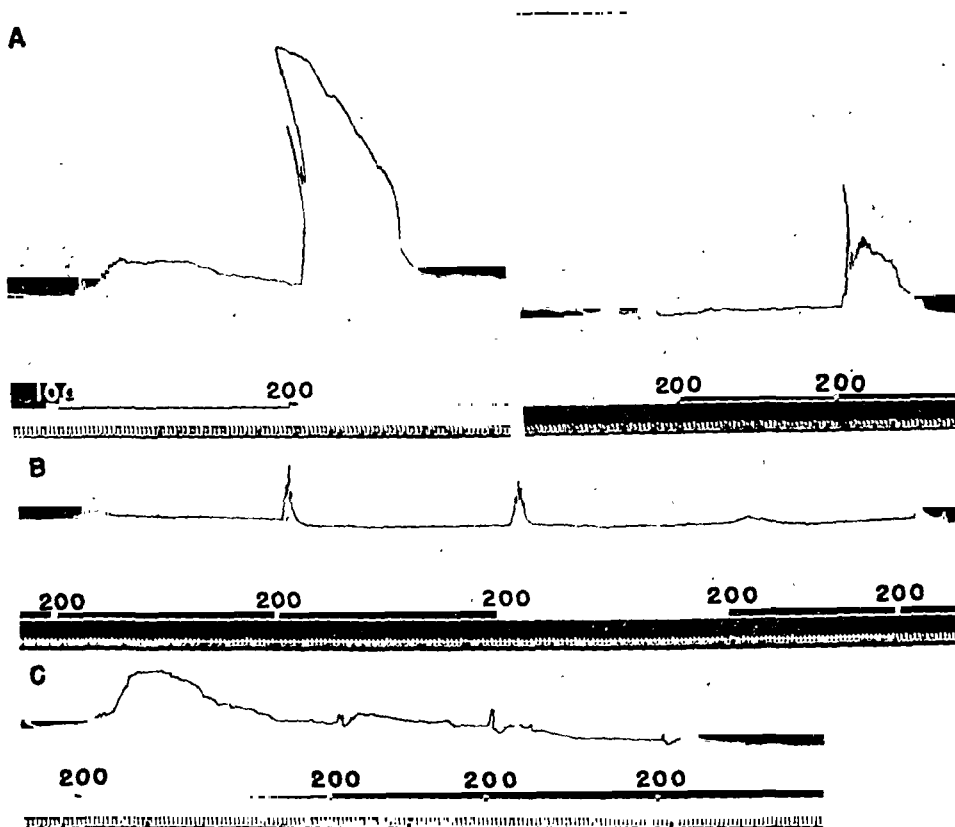


FIG. 3. CONTRACTIONS OF OPPOSING MUSCLE OF THE THUMB PRODUCED BY RAPID INJECTION OF ACETYLCHOLINE INTO RADIAL ARTERY AT WRIST

Doses are indicated in micrograms above the signal line. A, B, and C: three normal subjects. Between the two parts of A, there was a pause of a little more than a minute for readjustment of needle in artery. Bottom line: time in seconds and minutes.

fluid at approximately the same rate when these injections are repeated at intervals of 30 to 60 seconds.

A. Sensitivity to acetylcholine in myasthenia gravis. These results emphasize the difficulty in establishing a threshold dose of acetylcholine for stimulation of skeletal muscle with accuracy. Since there is so much variation in normal subjects, data on a statistically significant number of subjects would be needed to establish within reasonable limits a normal threshold. Even this normal value would not be useful in judging the acetylcholine sensitivity of a single myasthenic patient; only a value derived from a large group of myasthenic patients would be comparable to the statistically established normal value.

These considerations suggest how difficult it would be with the methods used to determine whether or not myasthenia gravis results from a

decrease in the sensitivity to acetylcholine. It seems likely in an all-or-none system like skeletal muscle that a slight change in sensitivity to the chemical mediator might lead to considerable weakness. A slight change in the threshold to acetylcholine would not be detectable by the methods thus far applied to the problem.

B. Variability of the response. What factors account for the variability of the response to the same dose of acetylcholine injected repeatedly into the same subject? The most difficult part of the manual technique of intra-arterial injection is the placing of the needle cleanly within the lumen of the artery and its maintenance in this position despite other necessary manipulations. But even when the manual technique was beyond reproach, striking differences occurred not only in the size of the motor response but also in the intensity of the sensory effects. There was no question of a

systematic decrease in the sensitivity of the muscles; as illustrated in Figure 3, the early responses were not necessarily the greatest.

The possibility must be considered that the amount of acetylcholine reaching the muscles was not uniform from injection to injection. This might result partly from slight changes in the streamline flow of the injected fluid in the arteries. Probably more important is the fact that acetylcholine is a vasodilator. Even doses too small to elicit contractions of skeletal muscles produced intense flushing of the skin supplied by the artery into which the injection was made. This kind of vasodilatation is accompanied by very large increases of blood flow through the skin, especially in the fingers. It is possible that much of the drug which might have reached the muscle was diverted to the skin or was shunted away from both skin and muscle through arterio-venous anastomoses. A further factor contributing to the variation among individuals is the variability of arterial supply to given muscular masses, particularly in the hand, where the volar arch permits a wide variation of supply as between radial or ulnar artery. No attempts to control these variables were made in the observations reported here.

C. Stimulation of skeletal muscle by acetylcholine. Brown, Dale, and Feldberg (4) established the fact that acetylcholine is capable of eliciting brief, twitch-like responses of skeletal muscle in experimental animals. In obtaining these responses; intravenous injection of acetylcholine was found to be quite ineffective, and even the injection into large arteries was an unreliable method. The best results were achieved by the injection of the acetylcholine into a relatively small artery near the muscle, occluding the artery proximal to the point of injection so that the injected substance would reach the muscle abruptly and relatively undiluted. This method the authors designated "close intra-arterial injection." In the experimental preparation which they used, certain volumes of injected fluid and certain rates of injection were optimal for the production of a twitch with the smallest dose of acetylcholine.

In general outline, the methods used here in man resembled the "close intra-arterial injection" used in experimental animals. Of the two arteries into which the injections were made in man, the radial is the smaller and the closer to the muscle from

which the records were made. The dimensions of the opposing muscle of the thumb are similar to those of the gastrocnemius muscle in the cat. Yet the radial artery supplies a considerable and variable part of the blood circulating the hand, and hence the acetylcholine is injected into larger and less uniform quantities of blood and vascular bed than is the case with the method of Brown, Dale, and Feldberg. When the injection is made into the brachial artery, the blood flow is even larger and the muscles studied have considerably greater mass and are more distant from the point of injection than with the radial artery. In the experiments reported here no occlusion of the artery was made during or after the injection.

The doses necessary to elicit these responses in man correspond to the probable conditions of blood flow, muscular mass, and distance of the muscle from the point of injection which were discussed above. Brown, Dale, and Feldberg found that 2.5 μ g. of acetylcholine were necessary to produce a small twitch-like response in the cat's gastrocnemius. When the radial artery was used in man, 30 or more μ g. were effective; and when the brachial artery was used, from 100 to 1000 μ g. produced small responses in different subjects. Previous investigators injecting acetylcholine rapidly into the femoral artery in the groin found no muscular contraction with ten times these doses (11, 12).

The contractions of skeletal muscles obtained in man on the whole resemble those found by Brown, Dale, and Feldberg in the cat. Some of the responses reported by these authors were almost as brief as the single twitch. In our experiments none of the responses to acetylcholine were so brief. Many of those observed in man, however, closely resemble the responses to acetylcholine illustrated in Figure 2 of the paper by Brown, Dale, and Feldberg. As in the cat, the twitches elicited by stimulation of the motor nerve were unaffected by these injections (Figure 2). As the dose of acetylcholine was increased, the duration of the muscular responses in man tended to increase until they were definitely more prolonged than those observed by Brown, Dale, and Feldberg.

When the myographic tracing indicated a prolonged contraction (see Figures 1, 2, and 3) it is not certain that all of this response was due to the action of the injected acetylcholine on the muscle

fibers. Often the tension of these muscles remained elevated for 15 to 45 seconds after the sensations which immediately followed the injection had disappeared. In some of these instances the subject felt that the muscle had relaxed to its original state while the myogram still indicated contraction. Since the sensory and motor connections of these muscles with the central nervous system were intact, it is possible that these prolonged contractions represented reflex changes of the tonic discharge of nerve impulses to these muscles or their antagonists. In Figure 1B it may be noted that the interposition of voluntary contractions or relaxations changed the succeeding basal tension of the muscles.

SUMMARY

1. Acetylcholine rapidly injected into the brachial artery at the elbow elicits contractions in the muscles of the forearm and hand. The threshold doses range between 0.1 and 1.0 mgm.

2. Five patients with myasthenia gravis did not differ from five normal subjects with respect to the character of these contractions or the range of threshold doses of acetylcholine.

3. Acetylcholine rapidly injected into the radial artery at the wrist elicits contractions of the opposing muscle of the thumb. The threshold doses range between 30 and 200 μ g.

4. Considerable variation of the muscular response occurs when the same dose is injected repeatedly at intervals of 30 to 60 seconds.

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RESPONSE OF CITRIC ACID LEVELS TO ORAL ADMINISTRATION OF GLUCOSE. I. NORMAL ADULTS AND CHILDREN¹

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It has been demonstrated in animal and plant tissue studies that citric acid metabolism is intimately related to carbohydrate metabolism (1-5). A direct relationship between glucose and citric acid metabolism in the human has not been established, hitherto.

A study was therefore undertaken to measure citric acid levels in the human, during enhanced activity in the oxidation of glucose and its conversion to glycogen. Two methods were employed. One method was the study of the changing citric acid levels in the blood serum simultaneously with the changing glucose levels during a glucose tolerance test. The other was to cause a rapid removal of glucose from the blood stream by injecting insulin without glucose administration into the normal adult.

Glucose (1 gm./kilo body weight) was administered orally after a 12-hour fasting period. Blood samples were drawn fasting, and one-half, one, two, three, four, five and six hours after administration of the glucose. All glucose determinations were done on serum by the method of Folin and Wu. Citric acid was determined by the methods previously described (6, 7). Determinations of citric acid by these methods are accurate to within $\pm 5\%$. The results obtained may be illustrated by a typical curve obtained on a normal adult (Figure 1).

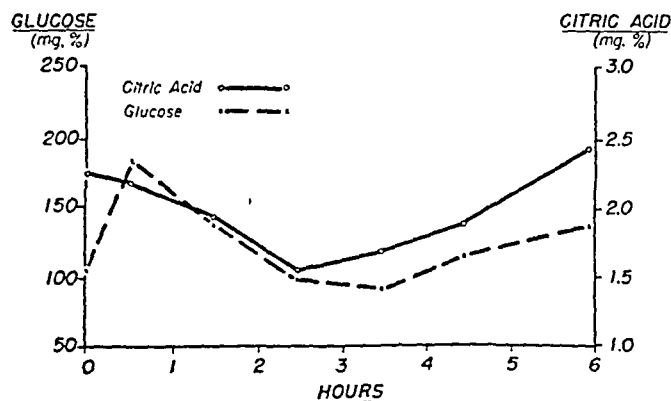


FIG. 1. CITRIC ACID LEVEL RESPONSE TO GLUCOSE ADMINISTRATION

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The characteristic glucose tolerance curve is observed in this case with a lowering of the glucose concentration to a value below the fasting level at the end of three hours and a return to normal at the end of six hours. The citric acid level drops slowly for the first 30 minutes and then drops sharply to a minimum point about 140-200 minutes after the administration of glucose. This minimum is from 25 to 35% lower than the fasting level. A gradual return to the fasting level is then observed. It is to be noted that the minimum for citric acid comes later than the glucose peak and corresponds with the time when one would expect

TABLE I

Adults			Children			
Case No.	Fasting level	Minimum	Case No.	Age	Fasting level	Minimum
	(mg. %)	(mg. %)		(years)	(mg. %)	(mg. %)
1	2.27	1.60	13	6	2.28	1.74
2	1.95	1.10	14	3½	3.66	2.40
3	1.78	1.20	15	4½	2.90	1.80
4	2.50	2.00	16	10½	3.40	2.40
5	2.60	1.70	17	12	3.20	2.05
6	2.23	1.54	18	1	2.47	1.88
7	2.62	1.60	19	2	2.32	0.80
8	2.80	2.30	20	4	2.65	2.08
9	3.03	2.46	21	4	2.67	2.15
10	1.90	0.80	22	14	2.00	1.54
11	3.00	2.45	23	1	3.12	2.47
12	2.47	1.67	24	2½	2.28	1.35
Average % drop = 29.8			Average % drop = 31.2			

most active rate of glucose removal. Table I summarizes the citric acid response for 12 normal adults and 12 normal children.

When insulin ($\frac{1}{2}$ unit/kilo) was injected subcutaneously into six consecutive non-diabetic patients and their blood levels taken one hour later, appreciable lowering of their citric acid level was observed in all cases. As can be seen from Table II, the drop was approximately 29% in citric acid level.

In a similar test, 20 units of insulin were in-

TABLE II

Serum level	Fasting	One hour after injection
	(mg. %)	(mg. %)
* Glucose	91	36
* Citric acid	2.1	1.5

* Average of six normal adults.

jected subcutaneously into a diabetic whose glucose level was at 400 mg. %. At the end of 45 minutes the glucose level had dropped to 300 mg. %. The citric acid level was lowered from an initial value of 3.4 mg. % to a final value of 2.7 mg. %.

In young children (8, 9) the fasting citric acid levels are generally higher than that found in adults (10-14). Normal infants show a delayed response for a period of up to one hour, but exhibit a normal minimum within two and one-half to three hours. Their recovery to normal levels is quicker, often being complete in four hours. Older children resemble adults in their response.

The citric acid fasting levels in newborns are higher than those observed in older children, ranging from 3 to 6 mg. %. Their citric acid response follows the pattern observed in normal infants but with a steeper initial drop. Simultaneously citric acid and glucose tolerance curves of a newborn infant in whom the initial feeding was the glucose tolerance test is shown in Figure 2.

DISCUSSION

It is apparent from the curves observed in the normal individual that the highest level of citric acid in the blood is the fasting level. Administration of glucose results in a drop in the citric acid level, which returns to the fasting level from five to six hours after administration of glucose. A relationship between glucose and citric acid metabolism is therefore demonstrated in humans.

The observations above coincide with the observations of Stoppani on depancreatized dogs. When glucose with insulin was injected into these dogs, a drop in the citric acid values was observed (15).

That insulin is the substance responsible for the drop in citric acid levels is not substantiated by our observations reported in the succeeding paper of this series (16).

Among the factors required to degrade glucose to pyruvic acid are phosphate ions, magnesium ions, adenosine triphosphate and coenzyme I (17). These same factors are needed for the formation of citric acid (18-21). One explanation for the dropping of the citric acid levels may therefore be one of inhibition by competition. The large requirements for these elements and coenzymes needed for the conversion of a large mass of glucose suddenly invading the blood stream, as in the glucose tolerance test, would decrease their availability for citric acid production. Phosphate ion is of particular importance for it is needed both for the phosphorylation of glucose and also for citric acid formation (18-21). Inorganic phosphate levels are lowered after insulin administration (22).

An alternative explanation may be based on the evidence that phosphorylation of glucose and glycogen formation require the utilization of the oxidative cycle as an energy source (23). Disappearance of citric acid from the blood stream may reflect this phenomenon.

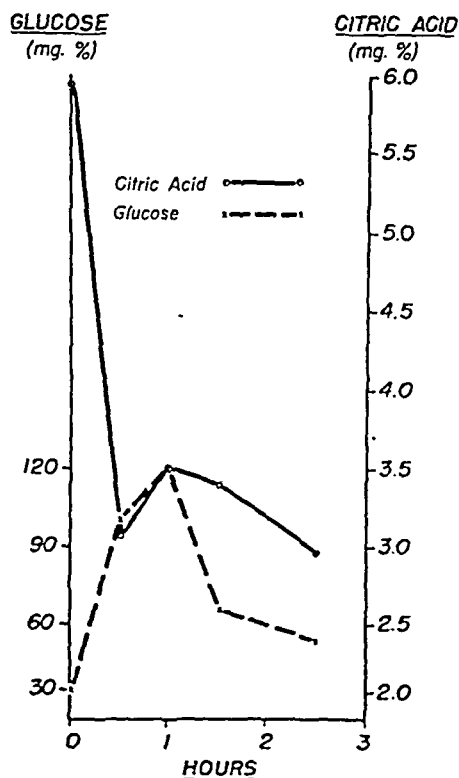


FIG. 2. EFFECT OF FIRST GLUCOSE FEEDING ON CITRIC ACID LEVELS IN NEWBORNS

Some support is found for these proposals in the fact that the citric acid level drops to its lowest level at the point where the glucose is disappearing most rapidly from the blood.

Another explanation which suggested itself as a cause for the lowering of citric acid in the blood on glucose administration to the normal was a lowering of the blood pH. That pH would have an effect was derived from the evidence which has been reported on the effect of the administration of acids and alkalies on citric acid levels.

It has been reported that administration of substances that tend to raise the blood and urine pH will increase the output of citric acid in urine and raise blood citric acid levels. Conversely, administration of substances which tend to lower the blood and urinary pH will cause a diminution in the output of citric acid in the urine and a lowering of the citric acid levels in the blood (24-39).

Hallman (40) in studies on tissue slices found that citric acid is produced best at an alkaline pH (7.5-8.0). Moreover, the activity of the enzyme citrogenase which catalyzes the formation of citric acid from acetoacetic and oxalacetic has its optimum pH in the alkaline range (41-43).

It would therefore appear that production of citric acid should be increased in alkalosis and decreased in acidosis.

In order to test this possibility, namely, that the lowering of citric acid levels during the glucose tolerance test may be an effect due to a lowering of pH, pH and CO_2 measurements were made during a glucose tolerance test. Actually a slight rise in pH (0.02 units) was observed, which was at its peak two hours after the administration of the glucose and corresponding to the minimum for the citric acid level. The pH returned to its original value after three hours.

The change in CO_2 content of the blood was negligible, dropping slightly to a minimum (2% below fasting) at a time corresponding to the minimum of the citric acid level, and returning to its original value at the end of the test. Other observers have reported either no change or a rise in pH after administration of glucose or insulin (44).

These observations eliminate the suggestion that acidosis was responsible for the drop in citric acid level during the glucose tolerance test.

SUMMARY

1. Administration of glucose orally brings about a characteristic changing level of blood citric acid in the normal individual. The citric acid level drops to a minimum (25 to 35% below the fasting level) and returns to the fasting level at the end of from five to six hours; a relationship is therefore indicated, in the human, between glucose and citric acid metabolism.
2. Injection of insulin alone into humans causes a lowering of citric acid levels.
3. The citric acid lowering in blood serum is greatest during the period when glucose is most rapidly disappearing from the blood.
4. In the normal individual the highest level of citric acid in the serum is the fasting level.
5. Various possible mechanisms for the citric acid level response to the oral administration of glucose are discussed.

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RESPONSE OF CITRIC ACID LEVELS TO ORAL ADMINISTRATION OF GLUCOSE. II. ABNORMALITIES OBSERVED IN THE DIABETIC AND CONVULSIVE STATE¹

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In the first paper of this series (1) it was demonstrated *in vivo* in humans that the normal response to active carbohydrate metabolism, as in the postabsorptive period for glucose, or after injection of insulin, is a lowering of blood citric acid levels.

In the studies presented below, the nature of the citric acid response curve in abnormal conditions is explored with the hope of throwing more light on the nature of the mechanism linking citric acid metabolism with glucose removal from the blood stream.

When abnormalities occurred they were apparent during the first three hours of this test. For this reason and for the convenience of the patient, we discarded the six-hour test and substituted a three-hour test, seeking the contour of the first half of the citric acid curve.

¹ Presented in part at the Meeting of the American Chemical Society, New York City, Sept. 15, 1947.

In order to see the effect of a disturbed insulin balance on citric acid levels we applied this study to diabetic adults. A large majority of diabetics exhibited a citric acid response which was not markedly different from that of the normal. A few typical examples are illustrated in Figure 1.

In some diabetics, however, the diabetic state was complicated by an abnormal citric acid response. This abnormality took the form of a rapidly rising level of citric acid which did not fall during the test period.

Another form of abnormality observed, was a sharp rise for the first hour followed by a lowering of the citric acid level to a minimum. These abnormal results were observed in cases of diabetes which were difficult to control with insulin therapy. These patients also had neurological symptoms, abnormal electro-encephalograms, and tended to go into insulin shock readily. The following curves obtained illustrate this type of response (Figure 2).

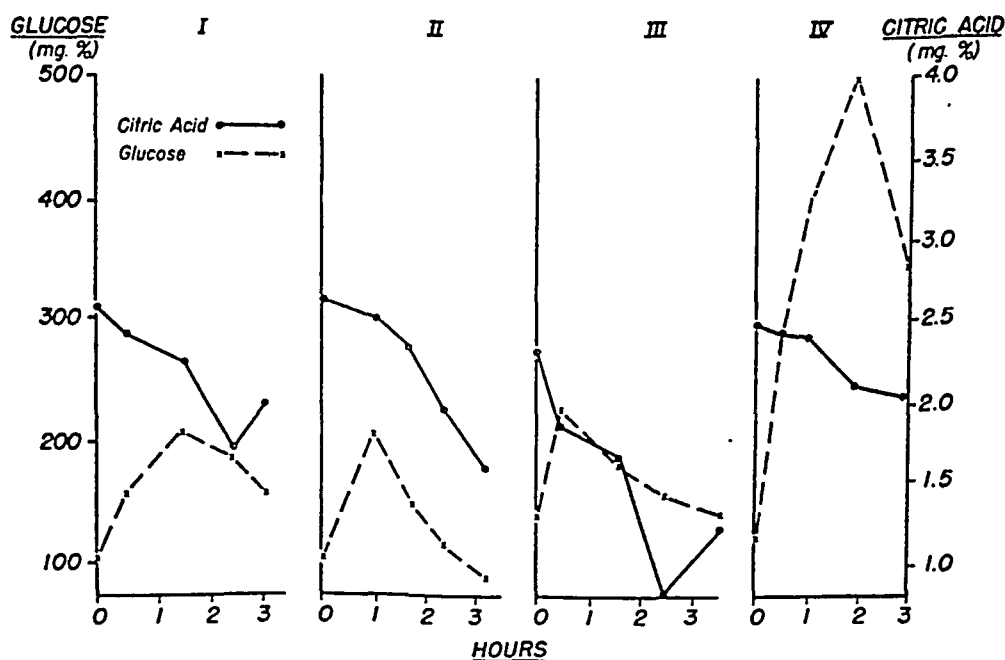


FIG. 1. DIABETICS WITH CITRIC ACID LEVEL RESPONSE RESEMBLING THE NORMAL

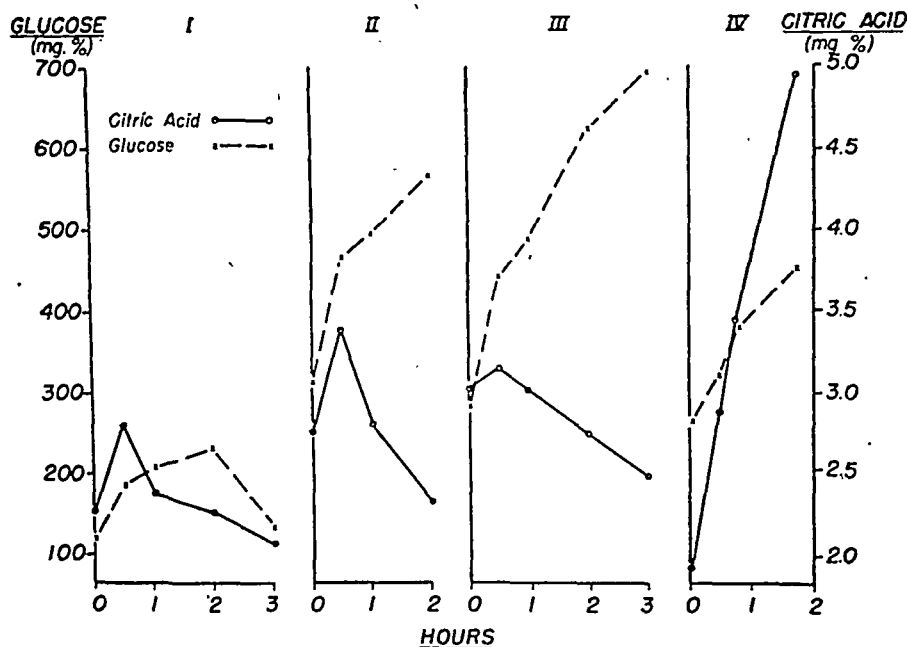


FIG. 2. DIABETICS WITH ABNORMAL CITRIC ACID RESPONSE

The rise of citric acid levels in certain pathological conditions raised the problem as to whether citric acid concentration would ever rise to a level where it would seriously affect the calcium ion concentration and, therefore, interfere with the normal action of calcium ion on muscle (2) and the oxidative system (3-7). It was therefore decided to study the citric acid response in children with symptoms of convulsions. Among the children studied in this group, six were encountered whose glucose tolerance curves were non-diabetic, but whose citric acid response was abnormal. This took the form of:

- (1) a continuous rise in citric acid levels during the test (Figure 3, Case I)
- (2) a rise followed by a delayed return to the fasting level (Figure 3, Cases II and IV)
- (3) a rise above the fasting level, followed by a lowering to a minimum below the fasting level (Figure 3, Case III).

The curves for four of these convulsives are shown in Figure 3.

One newborn child with typical symptoms of tetany (*i.e.*, carpopedal spasm, etc.), but with a total calcium not much below the normal range, was studied.

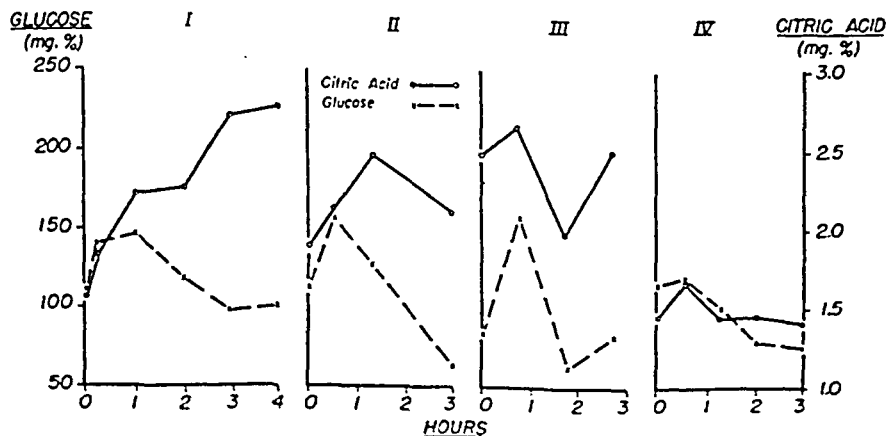


FIG. 3. CONVULSIVES WITH ABNORMAL CITRIC ACID RESPONSE

This child had a total blood calcium level of 8 mg. %, but a citric acid level of 9.7 mg. %. This would approximate an increase in citric acid of from about three to five times over the normal range. The calcium level of 8 mg. % could not explain the tetany. Calculations showed that if such a rise of citric acid concentration were to occur in the spinal fluid the increase in citrate level would cause a drop in calcium ion of 39%, which should be of physiological significance. These calculations were done for spinal fluid for it has been demonstrated that the calcium ion concentration in spinal fluid is controlled by the concentration of citrate alone, the protein effect being slight (8). Citric acid levels in spinal fluid are higher than that in the serum (9).

DISCUSSION

Figures 1 and 2 indicate that diabetics may be classified in accordance with their citric acid level response to administration of glucose.

It should be noted that the severity of the diabetic curve is no index of the citric acid response. In the diagrams above, Figure 2, Case I has a mild form of diabetes but an abnormal citric acid response. Case II exhibits a more severe form of diabetes and a response similar to Case I. Case III with severe diabetes exhibits a mild deviation from the normal. Case IV has a most abnormal citric acid response although her form of diabetes seems to be less severe than Case II or III. Case IV during her stay in the hospital was in shock or coma most of the time, and was admitted for a possible brain tumor, for which there were found to be negative clinical findings. This would indicate that the lowering of citric acid levels in the human on administration of glucose is not proportionately related to the patient's insulin response. Of pertinent interest is the observation of Stoppani that administration of glucose alone to depancreatized dogs causes a rise in citric acid levels (10).

It is probable that in severe diabetes a substantial portion of the citric acid in the serum rises from fat metabolism via acetoacetic acid with the aid of citrogenase in the presence of oxalacetic acid from the oxidative cycle (11-13). This mechanism would make it possible for citric acid levels to build up even though the formation of substantial

amounts of pyruvic acid were blocked by lack of insulin.

The curves for those convulsive children (Figure 3), where the glucose tolerance curves seemed to be essentially normal but the citric acid levels tended to rise during the tests, are of interest. These observations and the observations on diabetics with abnormal insulin-citric acid response may be interpreted as signifying that normal insulin equilibrium or abnormal insulin equilibrium is no indicator of the ability of the body to metabolize citric acid. Certain convulsive children exhibit evidence of abnormality in citric acid metabolism only. Abnormality in citric acid metabolism may, therefore, be interpreted as due to an abnormality in the function of the oxidative cycle.

The reasoning for this conclusion is based on experiments *in vitro* and *in vivo* with malonic acid, a specific inhibitor for the succinoxidase system. Addition of malonic acid to tissue *in vitro* will cause a rise in citric acid (14, 15). Injection of malonic acid into normal dogs causes a rise in citric acid serum levels (10). Thus, the condition in these patients with abnormal citric acid response might be compared to that in the animals with an inhibited oxidative cycle.

In the light of our studies it is suggested that a more intimate study of the oxidative cycle of patients with convulsive seizures of unknown origin and of diabetics with abnormal response to insulin therapy should be a fruitful method of attack on these problems.

Citric acid level response to oral administration of glucose may serve as an indicator in detecting *in vivo* changes of a portion of this system.

It has been demonstrated that the diffusible calcium changes in convulsions in children (16). That citric acid forms an un-ionized calcium complex is based on sound experimental evidence. Shelling and Maslow (17) demonstrated that in convulsions induced by administration of sodium citrate in massive doses, the diffusible calcium rose to a high level. They drew the conclusion that calcium forms a poorly ionized complex with citric acid. A number of years later Shear and Kramer (18) demonstrated by conductivity measurements that such was the case. In 1934 Hastings *et al.* (7, 19) studied this problem quantitatively. They drew the conclusion that calcium formed a poorly

dissociated complex with citric acid in the blood and spinal fluid. Similar observations were made with magnesium ions.

From the findings on the infant in tetany, one would expect that some cases of tetany may be explained by abnormally high citric acid levels. However, it must be stressed that this is probably not the case in the convulsive seizure as usually observed in convulsives. In these cases an impaired or inhibited oxidative system should be investigated as a possible cause.

SUMMARY

1. A study of citric acid levels in response to glucose administration was made in humans where abnormal glucose metabolism was suspected.

2. Most diabetics show no apparent deviation from the normal citric acid level response curve on administration of glucose orally.

3. Certain diabetics, with neurological symptoms, show abnormalities in their citric acid response to the administration of glucose.

4. Some children with symptoms of convulsive seizures show abnormal citric acid response curves, but non-diabetic glucose tolerance curves.

5. Citric acid levels can rise in pathological conditions to levels where calcium ion concentration can be significantly affected.

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THE EFFECTS OF VARIOUS AMINO ACIDS ON PERIPHERAL BLOOD FLOW AND SKIN TEMPERATURE¹

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Increases in skin temperature and peripheral blood flow following the ingestion of food have been reported by previous investigators (1 to 4). In 1941, Abramson and Fierst (5), using the venous occlusion plethysmograph, studied the effects of various dietary components upon peripheral blood flow in man. Following the ingestion of a predominantly carbohydrate meal, they found no significant change in total blood flow through the peripheral vascular beds of the hand, forearm or leg. Following the ingestion of a protein meal, however, an augmentation in blood flow became manifest in the hand, reaching its highest level 2 to 2½ hours after ingestion. A similar, though less marked, increase in flow was noted in the forearm and leg.

In view of the increase in oxygen consumption which occurs as a result of the specific dynamic action (S.D.A.) of protein, it might be postulated that the rises in skin temperature and blood flow subsequent to a protein meal bear a causal relationship to this phenomenon. Elevated skin temperatures in hyperthyroid patients have been observed by numerous investigators (6 to 8), and increases in blood flow have also been reported (9, 10). However, no consistent correlation between peripheral blood flow and basal metabolic rate was established by either Eichna and Wilkins (9) or Abramson and Fierst (10).

The S.D.A. of a protein is equal to the summed effects of its constituent amino acids (11). Recently, Gubner, DiPalma and Moore (12, 13) have reported increases in peripheral blood flow and skin temperature following the ingestion of glycine (amino-acetic acid). Although no quantitative parallelism between changes in oxygen consumption and peripheral blood flow were demon-

strated in their subjects, these authors concluded that the increase in blood flow resulted from the S.D.A. of glycine.

In an effort to determine the effects of various amino acids upon peripheral blood flow and skin temperature, and to examine the possible relationships between S.D.A. and the peripheral circulation, in the present study seven amino acids were administered to four healthy male subjects. S.D.A. is not influenced by the work of the digestive glands, intestinal movements or work of absorption from the intestines, since the effect of amino acids is the same whether they be administered orally, intravenously or subcutaneously (14, 15). For this reason, all of the amino acids used in these experiments were given orally. Oxygen consumption, skin temperatures, rectal temperature and blood flow through the hand were determined before and after ingestion of these compounds. The results obtained are presented in this paper.

METHODS

Measurements of skin and rectal temperatures, blood flow through the hand, and oxygen consumption were collected on four healthy, white male subjects whose ages ranged from 17 to 21 years. All studies were conducted in a constant temperature room with closed circuit ventilation and a turbulent air velocity of approximately three m.p.h. The experiments were carried out at three different ambient temperatures: 18° C., 24° C., and 30° C. The room temperature was controlled to within ± 0.5 C.° during any experimental day.

Skin temperatures were obtained with No. 30 B & S copper-constantan thermocouples connected to a Leeds and Northrup potentiometer. The thermal junctions were secured to the skin with small pieces of light adhesive tape and were placed as follows: forehead, right great toe, right forearm, abdomen, left thigh, and back. On the left hand ten thermocouples were connected in parallel (with six on the hand and four on the fingers) so that one temperature reading represented an average value for this hand. Rectal temperatures were recorded by means of a thermocouple at the tip of a No. 14 soft rubber catheter inserted at least 5 cm. above the internal rectal sphincter.

¹ Read at the symposium on military physiology, regional meeting of the American Physiological Society, Washington, D. C., December 6, 1947 (34).

² Formerly Captain, M.C.

Oxygen consumption was determined by gas analysis of samples of expired air which were collected in a 100-liter Tissot apparatus.

A hand plethysmocalorimeter (16) was used as an air plethysmograph and blood flow records were obtained by the venous occlusion method with a modified Brodie bellows. Each blood flow determination consisted of the average of seven consecutive flows taken at one-minute intervals. Flows were calculated in terms of cubic centimeters per 100 cubic centimeters of limb volume per minute.

On the night preceding an experiment, the subject was required to be in bed by 10 p.m. He was permitted no food or liquid other than water from this time until the end of the experimental period, the only material ingested being the particular compound studied that day.

The subject, wearing a light cotton undershirt, light cotton shorts, fatigue trousers, light woolen socks, and standard service shoes, entered the test chamber at 7:30 a.m. and remained at rest for approximately two hours. At the conclusion of this period, the skin and rectal thermocouples were put in place, the subject reclined comfortably in an adjustable chair and the left hand was inserted in the plethysmograph at heart level. Effort was made to allay apprehension and to minimize disturbing extraneous stimuli. Skin temperatures were measured until a steady state had been attained, i.e., until the average hand skin temperature remained unchanged within $\pm 0.2^\circ \text{C.}^\circ$ for no less than 40 minutes. When the steady state had been reached, blood flow records were obtained and the oxygen consumption was measured.

Following these procedures, the amino acid being studied was administered orally. Each drug was dissolved (or suspended when insoluble) in 300 cc. of unsweetened grapefruit juice at room temperature. The dosages were calculated in terms of grams of compound per pound of body weight. In the case of glycine, amounts ranging from 1 to 4 grams/10 lbs. of body weight were employed.³ Most of the other amino acids investigated were used in dosages of 1-2 grams per 10 lbs. body weight. The amino acids used were: glycine, dl phenylalanine, 1 (-) tyrosine, 1 (-) leucine, 1 (+) glutamic acid, 1 (+) histidine and dl methionine.⁴

Average hand skin temperatures were measured every ten minutes and all skin and rectal temperatures were recorded at 30-minute intervals. Blood flow through the left hand was usually determined at 30-minute intervals or whenever a significant change in hand skin temperature was observed.

Oxygen consumption was, in most cases, obtained approximately 80 minutes and 200 minutes following ingestion of the amino acid. Subjective reactions were obtained by questioning and were recorded throughout each experiment. Similarly, any visible flushing, perspiration, etc. were noted in the protocols.

³ For certain technical reasons, the dosages used have been expressed in terms of 1, 2, 3, and 4 grams per 10 lbs. body weight. These are equal to .22, .44, .66 and .88 grams per kilogram body weight, respectively.

⁴ The amino acids were kindly supplied by Merck & Co.

All experiments were continued for at least three hours following administration of the amino acid unless the subject complained of an uncomfortable subjective reaction.

Control studies, using 300 cc. of plain, unsweetened grapefruit juice, but otherwise identical in every respect to those described above, were conducted at each ambient temperature.

EXPERIMENTAL RESULTS

Glycine (aminoacetic acid)

Glycine was administered in dosages of 1 gram per 10 lbs. body weight (seven experiments on two men), 2 grams per 10 lbs. body weight (18 experiments on four men), 3 grams per 10 lbs. body weight (five experiments on two men), and 4 grams per 10 lbs. body weight (three experiments on one man) for a total of 33 experiments.

Subjective reactions

The principal adverse symptoms encountered following the oral administration of glycine were nausea and/or vague abdominal discomfort usually described as "a feeling of fullness." These reactions generally occurred immediately or within 15 minutes after swallowing the amino acid and, in most cases, disappeared within one hour. There were few complaints referable to the sweetish taste of the substance. Threshold for nausea varied from man to man but was quite constant for any one subject. Thus, Subject A experienced no discomfort from dosages up to and including 3 grams/10 lbs. body weight, but complained of severe nausea after the administration of 4 grams/10 lbs. body weight. Subject B was not adversely affected by 2 grams/10 lbs. body weight but was barely able to tolerate 3 grams/10 lbs. body weight. Subjects C and D invariably reported slight to severe nausea after receiving 2 grams/10 lbs. body weight. Four experiments, discontinued because of vomiting within two hours after administration of the drug, were discarded and are not included in the protocols.

On only one occasion was any subjective impression of increased warmth reported. This was accompanied by severe nausea and dizziness and occurred after a massive dose of glycine (4 grams/10 lbs. body weight). This was also the only instance in which any marked flushing of the face or extremities was noted.

Anorexia, lasting from three to eight hours after ingestion of the glycine, was a constant finding in all subjects. During the entire investigation, however, there was no significant weight change in any subject.

Studies at an environmental temperature of 24° C.

Skin temperature. At an environmental temperature of 24° C., ingestion of glycine in each of the four dosages employed caused a definite rise in skin temperature of the hand as compared with the controls. Once equilibrium had been reached, the hand skin temperature remained remarkably constant in the control series, the average maximum increase being only 0.4 C.° The average maximum increases in hand skin temperatures following the administration of 1, 2, 3, and 4 grams of glycine/10 lbs. body weight were 3.5 C.°, 4.8 C.°, 5.4 C.°, and 7.7 C.°, respectively. These results are highly significant from a statistical standpoint (variance analysis, Student's "t" test, Bartlett's test using chi-square), the probability being less than .01 that the differences between the control series and the glycine series were due to chance (17).⁵

⁵ The statistical analyses were made by Miss Agnes M. Galligan, whose aid is gratefully acknowledged.

Since 2 grams of glycine/10 lbs. body weight appeared to be the optimum dosage for producing peripheral vascular changes unaccompanied by nausea, the largest number of studies was conducted with this amount of the drug. A statistical comparison of the increments of hand skin temperature increases after glycine, 2 grams/10 lbs. body weight, vs. the increments of hand skin temperature increase of controls was carried out in an effort to determine the relationship between time of ingestion and increase in hand temperature. Skin temperature readings were compared at 20-minute intervals (Figure 1). No significant difference between the control and the glycine series could be demonstrated 60 minutes after ingestion. At 80 minutes, the controls showed an average decrease of 0.22 C.° and the glycine series showed an average increase of 0.49 C.°. This difference is significant, the probability being less than .05 that it could be due to chance. At 120 minutes the difference between controls and glycine was greater and the probability is less than .01 that the difference was due to chance. One hundred and eighty minutes after administration of the drug the difference between the control and the glycine series was most marked and statistically most significant.

AVERAGE SKIN TEMPERATURE, LEFT HAND OF FOUR SUBJECTS FOLLOWING INGESTION OF GLYCINE, 2 g./10 lb BODY WEIGHT

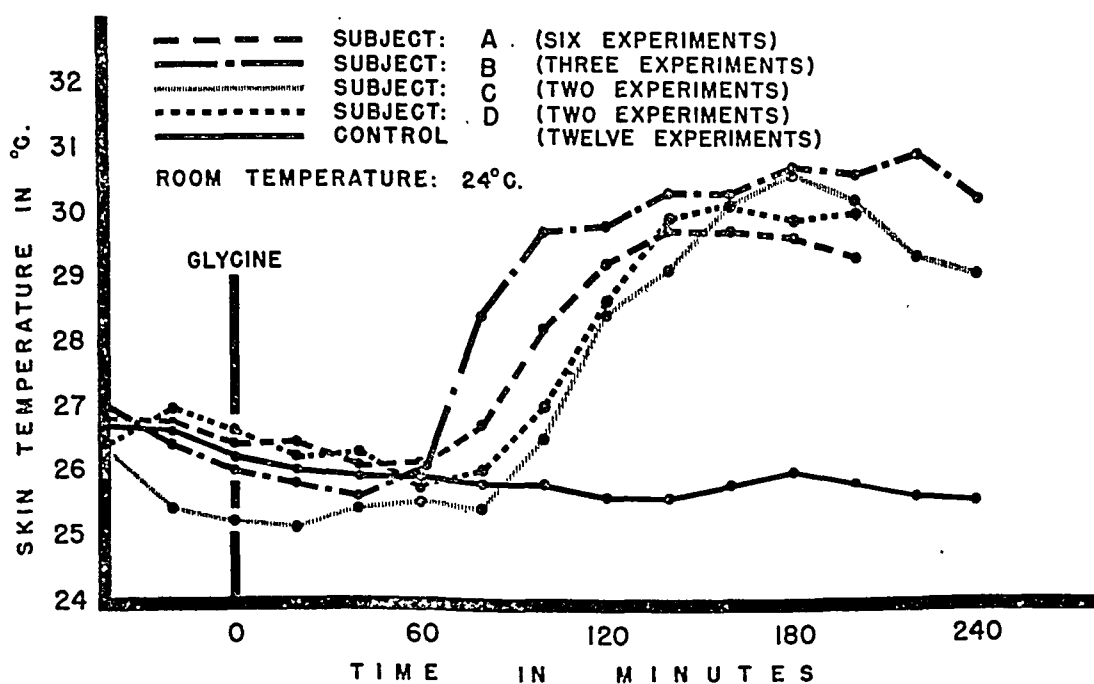


FIG. 1

A slight but significant increase in toe temperature occurred after the ingestion of glycine, 2 grams/10 lbs. body weight, as compared with the controls. Skin temperature of the right great toe *decreased* an average of 0.6 C.° for the control series while in the glycine series an average maximum *increase* of 1.8 C.° was observed. These figures were obtained by averaging the maximum increase (or minimum decrease) occurring 80 or more minutes after ingestion of glycine or, in the control series, 80 or more minutes after hand skin temperature had reached equilibrium.

The control and the glycine series demonstrated no significant differences in skin temperature of forehead, forearm, back, abdomen or thigh; nor were there any significant changes in rectal temperature.

Peripheral blood flow. At an environmental temperature of 24° C., ingestion of glycine in each of the four dosages employed, resulted in significant increase in blood flow through the hand as measured by venous occlusion plethysmography. The average maximum increases in flow following the administration of 1, 2, 3, and 4 grams of glycine per 10 lbs. body weight were: 1.6 cc., 2.3 cc., 3.6 cc., and 3.9 cc./100 cc. limb tissue/minute, the maximum amount of blood passing through the hand being approximately 2.5 to seven times the control flow. The statistical probability that these increases might have been due to chance is less than .01; the changes in blood flow paralleled the changes in hand skin temperature closely.

Oxygen consumption. Oxygen consumption, as determined by gas analysis of samples obtained from a 100-liter, open-circuit Tissot apparatus, increased after ingestion of glycine in 11 out of 12 experiments in which accurate measurements could be made. The changes in oxygen consumption ranged from 0 to 30% increase over the basal values. The rather marked variation between subjects and within the same individual in different experiments, is probably referable to (1) failure to attain a basal state initially, and (2) differences in the rate of absorption of the drug. With the optimum glycine dosage of 2 grams/10 lbs. body weight, the average rise in oxygen consumption was 13% at an environmental temperature of 24° C. Increases in oxygen consumption did not appear to be related significantly to the presence or absence of nausea.

Studies at an environmental temperature of 18° C.

Eight experiments were conducted on two men at an ambient temperature of 18° C. At this temperature, almost maximal peripheral vasoconstriction obtains in the lightly clad subject at equilibrium with the environment. Glycine, in the four dosages mentioned previously, was administered and the experiments were identical to those described above except for the different environmental temperature. A comparison of the glycine series with the control series revealed no significant differences in skin temperature or peripheral blood flow. In neither series did any rise in skin temperature or increase in blood flow through the hand occur. Oxygen consumption in the glycine series was usually greater than in the control series but the day-to-day variations were much more marked than at an environmental temperature of 24° C. This difference was probably due to fluctuations in muscular tensing caused by the cold stimulus. Gross shivering was never noted, although the subjects frequently complained of chilly sensations.

Studies at an environmental temperature of 30° C.

Glycine, administered in four experiments conducted on two subjects at an ambient temperature of 30° C., produced no rise in the already high skin temperature and no significant increase in peripheral blood flow as compared with the control series. No significant changes in rectal temperature occurred. Little or no change in oxygen consumption was observed in either series at this temperature.

dl phenylalanine

Because of its high S.D.A., the effects of phenylalanine on skin temperature and peripheral blood flow were of considerable interest. This amino acid, however, proved so distasteful to the subjects and produced such severe nausea that it was possible to complete only three experiments on three men. These were conducted at a room temperature of 24° C. In one of the tests an amount equal to only 0.5 gram/10 lbs. body weight could be swallowed by Subject C. This produced an increase in hand skin temperature equal to 1.9 C.° but no appreciable rise in blood flow through the hand. Subjects A and B were able to ingest 1 gram/10 lbs. body weight and 1.3 grams/10 lbs.

body weight, respectively. The former dose produced a maximal hand skin temperature increase of 3.9 C.° and resulted in a blood flow through the hand equal to 4.8 times the basal flow (an increase of 3.0 cc./100 cc. limb volume/minute). The latter dose caused an increase of 7.2 C.° in hand skin temperature and increased the peripheral blood flow 4.1 times (an increase of 3.1 cc./100 cc. limb volume/minute). There was also a slight but significant increase in toe temperature. Phenylalanine, in a dosage of 1.3 grams/10 lbs. body weight, resulted in a 6% rise in oxygen consumption.

In these three experiments no effects on either rectal temperature or skin temperature elsewhere in the body were observed following ingestion of phenylalanine.

l (+) glutamic acid, l (+) histidine monohydrochloride, l (-) tyrosine, l (-) leucine, dl methionine

Glutamic acid was used in five experiments conducted on two subjects at an environmental temperature of 24° C. The dosages employed were 1 gram/10 lbs. body weight (two experiments), 1.5 grams/10 lbs. body weight (one experiment), and 2 grams/10 lbs. body weight (two experiments). No statistically significant changes in skin temperatures, rectal temperatures or blood flow through the hand occurred after administration of this substance. Oxygen consumption was increased an average of 8% over basal levels following ingestion of 2 grams/10 lbs. body weight.

One man, Subject D, was given histidine on three occasions in dosages of 0.5 grams, 1.5 grams, and 2 grams/10 lbs. body weight at an environmental temperature of 24° C. Very slight, and questionably significant, rises in hand skin temperature and peripheral blood flow were observed following ingestion of the larger doses. A comparable increase in toe temperature also occurred. No significant changes in rectal temperature or in skin temperatures elsewhere were noted, nor was there any increase in oxygen consumption. In the four experiments in which tyrosine, leucine, and methionine were administered (room temperature 24° C.), no effects on skin temperature, rectal temperature or peripheral blood flow were demonstrated.

Relationship between total oxygen consumption, blood flow through the hand and skin temperature of the hand

No consistent relationships between either total oxygen consumption and hand skin temperature or total oxygen consumption and peripheral blood flow were observed in this investigation. High skin temperatures and flows occurred as frequently with low oxygen consumption values as with high ones and vice versa.

The data were analyzed to determine the possible existence of a time factor. Skin temperatures and blood flows were compared (1) at the time of maximum increase in oxygen consumption and (2) at 30-minute intervals before and after the maximum increase in metabolism. No clear-cut relationship was demonstrated in either case. Total heat production was also measured in the control series. In these experiments blood flow and skin temperature remained quite constant, though oxygen consumption varied considerably from day to day for a particular individual. Here again, no relationships between metabolic activity and blood flow or skin temperature were noted.

At an environmental temperature of 24° C., a highly significant correlation of .88 was found to exist between hand skin temperature and blood flow through the hand. The curve expressing the relationship between these two variables tends to become asymptotic at a skin temperature of 34° C. (Figure 2).

DISCUSSION

These studies were initiated in an attempt to determine quantitatively the increase in peripheral blood flow and skin temperature reported to occur as a result of S.D.A. (12, 13). Of the seven amino acids used, five possess high S.D.A. (glycine, phenylalanine, tyrosine, glutamic acid, and leucine). Although no consistent relationships between S.D.A. and peripheral blood flow were observed, it should be emphasized that the lack of correlation between cellular energy exchange and peripheral blood flow in these studies applies only to relatively small changes in total oxygen consumption. Under circumstances in which massive metabolic differences are induced, a significant relationship may well obtain.

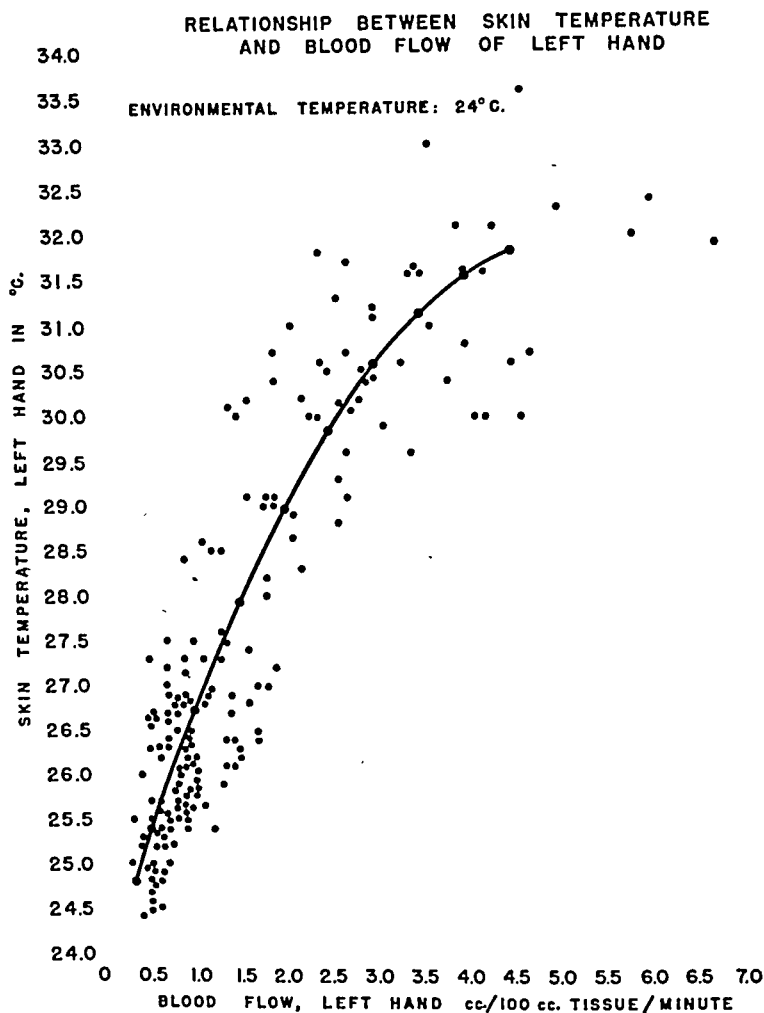


FIG. 2

That the ingestion of glycine exerts an effect upon the peripheral vascular bed under certain conditions, seems evident from this study. If this effect is not caused by the S.D.A., however, to what can it be attributed? The metabolism of glycine differs from that of other amino acids in several important respects. Gutman and Alexander (18), using specific methods for the determination of glycine, administered the amino acid to 40 subjects in dosages of 1 gram per 10 lbs. body weight. They found that, following ingestion of this substance, its concentration rises rapidly in plasma and erythrocytes, attaining its highest peak in from one to one and a half hours. The speed with which ingested glycine is absorbed into the blood stream is interesting, but the slowness

with which the blood stream is completely cleared of ingested glycine is more remarkable and stands in contradistinction to the behavior of other amino acids tested, which are rapidly removed (19, 20). Its accumulation in other tissues is also unique. It has been demonstrated that glycine is the only amino acid which causes an increase in the amino nitrogen of muscle, as well as a greater rise in liver amino nitrogen than any other amino acid (21). The persistence of this substance in the body may well be due to a reduction in glomerular filtration and a high rate of renal tubular reabsorption for glycine (22, 23). It is possible that the peripheral vascular effects of glycine may be related in some manner to the uniquely high amino nitrogen content of muscle which occurs following

ingestion of this substance and to the extremely slow rate at which it is cleared from the blood and tissues.

In these experiments, the only sites at which skin temperature rises occurred after the administration of certain amino acids were the hands and toes. The vascular anatomy of these two areas is different from the peripheral vascular bed elsewhere in that arteriovenous shunts are present in the toes, fingers, and thenar and hypothenar eminences (24). Differences between the physiological reactions of blood vessels at these sites and the peripheral vascular bed in other portions of the body have been demonstrated by numerous investigators (25 to 29).

It should be noted that the slight rises in toe temperature are probably more significant than the absolute figures indicate since in almost all experiments the toe was still cooling down when the amino acid was administered. Because of this, the "equilibrium temperature" assumed for the toe was usually somewhat higher than the true equilibrium temperature.

The failure of glycine to produce any changes in peripheral blood flow or skin temperature at low (18° C.) ambient temperatures is in agreement with the findings of Ferris and his associates, who demonstrated that under cold ambient conditions it is extremely difficult to induce vasodilatation (30). It is evident that, in the present investigation, the neurogenic stimulus of cold was preponderant and could not be overcome by glycine. At an ambient temperature of 30° C., blood flow and skin temperature were extremely high before the glycine was administered and very little additional vasodilatation could be effected. Similar observations have been made with other vasodilator drugs (31, 32).

It is the opinion of the author that glycine is of little therapeutic value as a vasodilating agent. In our experience, direct or indirect vasodilatation in the extremities can most easily and effectively be achieved by the application of heat to various body areas (33).

SUMMARY

1. Skin and rectal temperatures, total oxygen consumption, and blood flow through the hand have been studied in four healthy young males

before and after the oral administration of various amino acids.

2. At an environmental temperature of 24° C., ingestion of glycine caused a significant rise in skin temperature of the hand. In these experiments the average maximum increases in hand skin temperature following the administration of 1, 2, 3, and 4 grams of glycine/10 lbs. body weight were 3.5 C.°, 4.8 C.°, 5.4 C.°, and 7.7 C.°, respectively. Hand skin temperature rises were accompanied by similar, though less marked increases in toe temperature.

3. At an environmental temperature of 24° C., ingestion of glycine in the four dosages employed, resulted in a significant increase in blood flow through the hand as measured by venous occlusion plethysmography. Average maximum flows amounted to 2.5 to seven times the control flow.

4. The increases in blood flow and hand skin temperature generally became apparent about 80 minutes after ingestion of glycine and were most marked approximately 180 minutes after ingestion.

5. No significant changes in rectal temperature or skin temperatures elsewhere in the body were observed.

6. At environmental temperatures of 18° C. and 30° C., no significant changes in rectal temperature, skin temperatures, or peripheral blood flow occurred after the administration of glycine.

7. At an environmental temperature of 24° C., ingestion of dl phenylalanine effected increases in hand and toe temperatures and blood flow through the hand similar to those observed with glycine.

8. At an environmental temperature of 24° C., oral administration of 1 (+) histidine monohydrochloride resulted in very slight and questionably significant increases in hand skin temperature and blood flow through the hand.

9. Ingestion of 1 (+) glutamic acid, 1 (–) tyrosine, 1 (–) leucine and dl methionine had no effect upon rectal temperature, skin temperature or peripheral blood flow.

10. Although five of the seven amino acids used in these studies caused a definite increase in oxygen consumption, no consistent quantitative relationships between total oxygen consumption and skin temperature or total oxygen consumption and peripheral blood flow were demonstrated.

ACKNOWLEDGMENT

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STUDIES OF THE MUCIN-CLOT PREVENTION TEST FOR THE DETERMINATION OF THE ANTIHYALURONIDASE TITRE OF HUMAN SERUM¹

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INTRODUCTION

The presence in human serum of an inhibitory substance which is capable of neutralizing an enzyme elaborated by a strain of hemolytic streptococcus recently has been demonstrated by Friou and Wenner (1). They further showed that the amount of inhibitory substance in sera from patients with rheumatic fever was greater than in sera from patients early in the course of uncomplicated hemolytic streptococcal infections or from normal individuals. These workers (1) employed the mucin-clot prevention test (M.C.P.) described by McClean (2) which was based on the observations of Seastone (3) and Meyer and Palmer (4) that hyaluronic acid mixed with serum protein in the presence of acetic acid gives a characteristic clot.

A brief historical background of the work preceding that of Friou and Wenner begins in 1928 with the observation of Duran-Reynals (5, 6) that vaccinal infection of the rabbit was considerably enhanced when the virus was injected into the skin along with an aqueous extract of rabbit, guinea pig or rat testicle. McClean (7) and Hoffman and Duran-Reynals (8, 9) in 1930 described independently the ability of testicular extracts to increase tissue permeability. These extracts were subsequently termed "spreading factors." Extensive studies by Duran-Reynals later showed that spreading factors were present in extracts of certain invasive bacteria (10), poisonous insects and snake venoms (11). Meyer *et al.* (12) found similar so-called enzymes in autolysates of a rough (R) type II pneumococcus. Enzymes of the same type were subsequently obtained from the bovine ciliary body and iris (13), from certain strains of Group A hemolytic streptococcus and spleen (14, 15), and from *Clostridium welchii* (10, 16).

It remained for Chain and Duthie (17, 18) in 1939 to show the relationship between these enzymes (hyaluronidases) and the spreading factor and to correlate the phenomenon of spreading in animal tissues with an enzymatic effect on the hyaluronic acid of connective tissue.

Recently studies dealing with the composition of connective tissue has received deserved attention. In 1934 Meyer and Palmer (19) reported the occurrence in vitreous humor of cattle eyes of a mucopolysaccharide consisting of equimolecular concentrations of acetylglucoseamine and glucuronic acid. They named this substance hyaluronic acid. In the same year Bensley (20) reported on histological observations of connective tissue. The distribution of ground substance was found to be the same in the umbilical cord, intima and media of blood vessels, connective tissue of lower vertebrates, gastric mucous membranes, and in general in all reticular and embryonic tissues. Meyer *et al.* have since extracted hyaluronic acid from aqueous humor of cattle (21), Wharton's jelly (12), and synovial fluid (22). It has also been extracted from rabbit and pig skin (23, 24), rabbit fascia (16) and the capsules of certain strains of hemolytic streptococci (14, 25).

Duran-Reynals (26) in 1932 reported experiments which showed that the spreading factor of testicle was neutralized *in vitro* by an antiserum against homologous testicle extract. McClean (27) and McClean and Hale (28) have demonstrated that sera prepared against the so-called spreading factor, neutralizes the diffusing, viscosity reducing, and hydrolytic activity of *Clostridium welchii* and vibron septique. They also demonstrated antihyaluronidase activity against the homologous enzyme in rabbits immunized with hyaluronidase from Group A. type 4 and Group C. type 7 streptococci.

These studies and work now in progress (29) indicate that streptococcal hyaluronidase is anti-

¹ Aided by a grant from the Life Insurance Medical Research Fund.

² Milbank Memorial Fund Fellow, 1946-47.

genic in rabbits and that antisera can be produced against this enzyme. There are, however, some different opinions concerning the specificity of these antienzymes. Haas (30) states that there is an enzyme (antinvasin I) in normal blood plasma of all mammals, birds, and fish investigated by him, which, by destroying hyaluronidase, acts as an anti-invasive catalyst. According to Haas this substance destroys hyaluronidases derived from a number of different sources. Following their recent studies, Hechter and Scully (31) cast some doubt in the biological significance of the enzymatic theory for the mechanism of invasion proposed by Haas.

The purpose of this paper is to describe studies of the method of antihyaluronidase determination by use of the mucin-clot prevention test (M.C.P.), first used by Robertson, Ropes, and Bauer (16) and later standardized by McClean (2).

MATERIALS

Enzyme:

The enzyme used in these tests was obtained from a strain of Group A, type 4 streptococcus hemolyticus. 1000 ml. of beef heart infusion broth enriched with 10% normal calf serum was seeded with 25 ml. of a 12-hour beef heart infusion broth culture of Group A, type 4 hemolytic streptococcus and incubated at 37° C. for 36 hours. The bacterial cells were separated from the supernatant fluid by centrifugation and the supernatant fluid which contained the enzyme was then filtered through a Seitz filter and the enzyme content titrated. This supernatant fluid was kept frozen at -70° C. in 40-ml. lusteroid tubes until ready for use as enzyme. Two lots of enzyme, A and B, were prepared; the enzyme content of each lot was found to be similar.

Substrate:

Potassium hyaluronate was prepared from human umbilical cords according to the method of McClean *et al.* (30). The cords were washed free of blood and stored in acetone in the refrigerator until ready for use. The cords were then ground in a meat grinder. To 500 grams of the minced cords were added 1000 ml. of water and the mixture allowed to stand for 24 hours in the refrigerator. The mixture was then squeezed through four thicknesses of surgical gauze. The mince was again extracted with 1000 ml. of water. The pooled extracts were centrifuged at 2200 RPM for 30 minutes. The thick, grayish-pink viscous solution was adjusted to a pH between 9 and 10 with potassium hydroxide. One and one-fourth volumes of cold methyl alcohol saturated with potassium acetate were added slowly to these pooled extracts which had previously been cooled in the refrigerator. The potassium hyaluronate which was precipi-

tated as a characteristic asbestos-like clot was washed several times with cold methanol, then with cold ether and finally dried *in vacuo* over phosphorous pentoxide. It was then ground into a fine powder and stored at room temperature until used. It was found that a greater yield could be obtained during each extraction if the acetone in which the umbilical cords were kept was changed at least once. A 0.15 per cent solution of potassium hyaluronate in distilled water was made up in 100-cc. volume and stored in the refrigerator at -4° C. This solution was used within three to four days and was sufficient to test 40 sera. No preservative was employed. Solution of the powder was facilitated by placing the flask in an incubator at 37° C. for two to three hours with occasional shaking. It is advisable to make up several grams of potassium hyaluronate and use the same hyaluronate for all experiments rather than use hyaluronate extracted at different times, since the strength of the hyaluronate may vary with each extraction and thus introduce another variable into the test. McClean (2) showed that the apparent potency of enzyme is inversely proportional to the concentration of the substrate mixture. Although different preparations of potassium hyaluronate were used in these experiments the enzyme titre did not change more than one two-fold dilution when the same enzyme was tested against substrate containing potassium hyaluronate extracted at different times. The potassium hyaluronate was standardized by testing the unknown preparation of potassium hyaluronate with a constant amount of purified hyaluronidase (bull's testicle, Schering). The clotting ability of different hyaluronate solutions used in the substrate was adjusted to give the same end point with a standard amount of hyaluronidase by dissolving the proper weight of potassium hyaluronate in water. This amount was usually 1.5 mgm. per ml. of water.

Serum:

The blood used in the study of normal individuals and patients was drawn as aseptically as possible. The serum was separated from the clot within 24 hours in most instances, and stored in lusteroid tubes at -70° C.

Normal horse serum (Lederle) was used in the substrate. Potassium hyaluronate will not form a clot on the addition of acetic acid unless blood serum is also present. Normal rabbit serum and calf serum were tried with equally good results. None of these sera contained antihyaluronidase when tested by the mucin-clot prevention test.

Preparation of substrate mixture:

In each tube in the test, 1 ml. of substrate was used. The total volume was made up in the following proportions:

Substance	Ratio
Potassium hyaluronate 0.15% in distilled water	1
Normal Horse Serum 1:10 dilution in Normal saline	1
Distilled water	2

To avoid waste and diminished clot-forming ability of the substrate it was found best to make up only enough substrate for each day's testing.

EXPERIMENTAL METHODS

The mucin-clot prevention test was used as a test for hyaluronidase and antihyaluronidase. The method used is a modification of that described by McClean, Rogers, and Williams (32) which was employed by Friou and Wenner (1). The serum being tested rather than the enzyme was the variable factor in the test. Serum dilutions were made in two-fold decrements and each serum was tested against a constant amount of hyaluronidase.

A. Test for hyaluronidase

1. Serial two-fold dilutions of the enzyme being tested were made in distilled water; 0.5 ml. to each tube.
2. 0.5 ml. distilled water was added to each tube.
3. The racks of tubes were placed in an ice bath for five minutes and then 1.0 ml. of substrate was added to each tube. (An automatic pipette greatly facilitates the speed and accuracy of adding solutions to the tubes.)
4. The racks were placed in a water bath at 37° C. for 20 minutes.
5. The racks were then removed and placed in an ice bath for five minutes.
6. Finally 0.2 ml. of 2 N acetic acid was added to each tube beginning with the greatest dilution and proceeding to lesser dilution and the tubes were shaken lightly.

Figure 1 shows an enzyme titration and the determination of enzyme units. Frequent titration of the enzyme

revealed it to be quite stable. Its titre did not change in a 14-month period.

Occasionally when a tube is near the end point of the enzyme, shreds will form. In these studies only a fully formed clot was counted as +. Shreds were counted as 0. A photograph of four tubes of a completed ten-tube test for hyaluronidase overemphasizes the turbidity of the solution. In the actual test turbidity is much less noticeable and the clots are seen more clearly. Following the lead of McClean, Rogers, and Williams (32) results were expressed in terms of the original dilution of enzyme in 1.0 ml. and the subsequent dilution with substrate mixture was disregarded. Data in Figure 1, where a clot is first seen in the tube in which the final enzyme dilution before addition of substrate is 1:2048, are interpreted to mean that a 1:1024 dilution of the enzyme being tested is just enough to prevent the formation of a clot in the substrate on the addition of 0.2 cc. of 2 N acetic acid. The amount of enzyme in the last tube (next to the end point) has been arbitrarily designated as 1 unit. 16 units of this particular enzyme would be a 1:32 initial dilution or 1:64 final dilution.

B. Test for antihyaluronidase:

1. Serial two-fold dilutions of the patient's serum were made in distilled water, 0.5 ml. to each tube. It was found that the end point of most sera would fall within a ten-tube range if the first tube contained a 1:32 dilution of the serum under test. Occasionally the antihyaluronidase titre of a serum will be too low or too high to fall within this ten-tube range. Then, a further titration must be done using lower or higher dilutions of serum

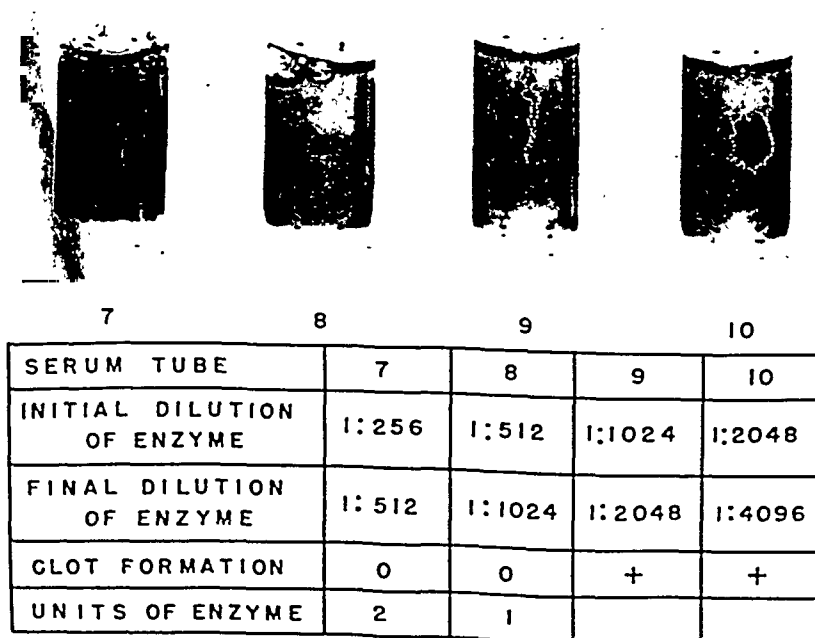
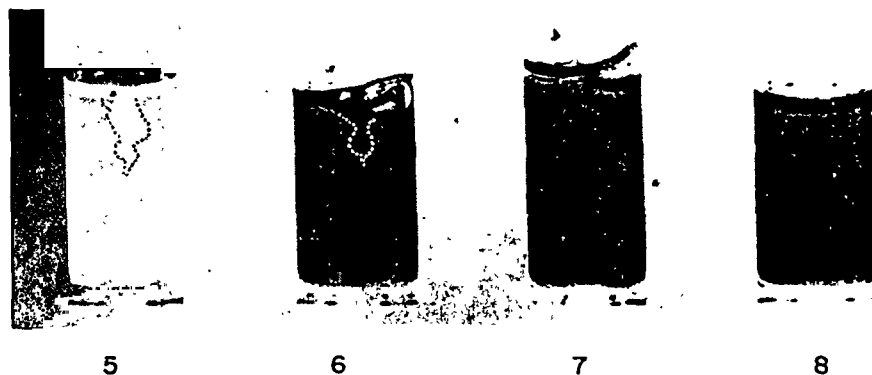


FIG. 1. SAMPLE TITRATION OF HYALURONIDASE.

**ENZYME SOURCE--STREPTOCOCCUS HEMOLYTICUS
GROUP A--TYPE 4**

INITIAL DILUTION OF ENZYME1:32
FINAL DILUTION OF ENZYME.....1:128
UNITS OF ENZYME.....16



SERUM TUBE	5	6	7	8
INITIAL DILUTION OF SERUM	1:512	1:1024	1:2048	1:4096
FINAL DILUTION OF SERUM	1:1024	1:2048	1:4096	1:8192
CLOT FORMATION	+	+	0	0

FIG. 2. TITRATION OF A SERUM FOR ANTIHYALURONIDASE

as the case may be. Usually 12 to 20 sera were tested at a time.

2. 0.5 ml. of a predetermined dilution of enzyme was added to each of ten tubes and the racks were allowed to stand at room temperature for 15 minutes. The enzyme dilution was adjusted so that the same amount of enzyme was used in each test. Most of the sera were tested against 16 units (0.5 ml. of an initial dilution of 1:32) of enzyme. Occasionally 32, 64 or 128 units of enzyme were used.

3. The racks were then placed in an ice bath and allowed to remain there for five minutes.

4. 1.0 ml. of substrate was then added to each tube and the racks were placed in a water bath at 37° C. for 20 minutes.

5. The racks of tubes were then removed and placed in the ice bath for five minutes.

6. 0.2 ml. of 2 N acetic acid was finally added to each tube beginning with the greatest dilution and proceeding to the lesser dilution and the tubes shaken gently. (Figure 2 shows the results of the determination of the anti-enzyme titre of a serum.) The first tube in which a clot appeared reading from greater to lesser dilution was established as the end point of the serum antienzyme titre.

The serum represented in Figure 2 was capable of inhibiting 16 units of streptococcal hyaluronidase in a final dilution of 1:2048. The antihyaluronidase titre of this serum therefore is 1:2048 when tested against 16

units of enzyme. Here again the photograph overemphasizes the turbidity of the solution. Precedent for not including the volume of the substrate mixture in calculating the antihyaluronidase titre can be found in the work of McClean (2).

7. Along with the unknown sera two known sera were tested. These sera served as controls. It is important to make serum dilutions with fresh serum each day. If sera are previously diluted and stored in an ice box the antienzyme titre decreases markedly within a few days.

RESULTS

The length of time of incubation of the enzyme-substrate mixture at 37° C. was 20 minutes in all the tests for the determination of hyaluronidase. However, hydrolysis of the substrate by the enzyme continues rapidly for at least an hour according to McClean's (2) work. In Table I is shown the results of incubating the enzyme-substrate mixture for variable periods of time. The hyaluronidase titre obtained was the same after five- and 20-minute incubation periods but the titre was slightly higher after 60 minutes of incubation.

The two lots of hyaluronidase used throughout these experiments were of almost equal strength.

TABLE I

The effect of incubation time on the enzyme strength
 Test for Hyaluronidase
 Source of Enzyme: Streptococcus Hemolyticus
 Group A—Type 4

Time of incubation of enzyme-substrate mixture	Final enzyme titre
5 minutes	1:1024
20 minutes	1:1024
60 minutes	1:2048

The results of duplicate titrations of enzyme performed on different dates are shown in Table II, and they suggest that a more accurate titration of the enzyme can be made if the enzyme dilutions are made in increments of 50 or 100 in each successive tube, *e.g.*, 1:50, 1:100, 1:150, 1:200, etc. rather than in serial two-fold dilutions.

TABLE II

Methods of dilution of enzyme to determine its strength
 Tests for Hyaluronidase in Duplicate
 Source of Enzyme: Streptococcus Hemolyticus
 Group A—Type 4

Date of tests	Final enzyme titre		
	Increment of 50	Increment of 100	Serial two-fold dilution
17 June 1947			
1st Test	1:900	1:800	1:1024
2nd Test	1:800	1:600	1:1024
18 June 1947			
1st Test	1:900	1:600	1:1024
2nd Test		1:600	1:1024
1 July 1947			
1st Test	1:800	1:800	1:1024
2nd Test	1:800	1:800	1:1024

The enzyme preparations did not lose strength when stored in a refrigerator at -4°C . or in the frozen state at -70°C . Titrations of the same enzyme several months apart revealed the same titre.

Apparently the reaction between the inhibitory

TABLE III

Test for antihyaluronidase to demonstrate the effect of length of the time of incubation of the serum-enzyme mixture on the antienzyme titre

Enzyme Source: Group A, Type 4 Hemolytic
 Streptococcus 64 Units

Time of reaction between serum and Enzyme at room temp.	Serum antienzyme titre
5 minutes	1:128
15 minutes	1:128
20 minutes	1:128
190 minutes	1:512

substance in serum and the streptococcus enzyme was nearly completed within five minutes. Table III shows the results of a test to demonstrate the effect of the length of time of incubation of the serum-enzyme mixture on the antihyaluronidase titre. Incubation of the mixture for 190 minutes resulted in a four-fold increase in the final antihyaluronidase titre as compared to the titre obtained after only five minutes' incubation. There was no difference in the antihyaluronidase titre obtained after incubation periods of five, 15, and 20 minutes.

In order to determine the effect of different storage temperatures on the antihyaluronidase titre of sera, samples of sera were stored at -70°C ., -4°C ., and at room temperature. There was no apparent loss of titre in sera kept frozen at -70°C ., but in serum stored at room temperature the antihyaluronidase titre decreased considerably. The titre of serum stored at -4°C . decreased slightly (Table IV).

TABLE IV

Effect of storage temperature and time on the antihyaluronidase titre of sera

Serum	Storage temperature and time			
	-70°C . 13 Mo. antihyalu- ronidase titre	-4°C . 2 Mo. antihyalu- ronidase titre	Room Temp. 2 Mo. antihyalu- ronidase titre	Original antihyalu- ronidase titre
A	1:32768	1:16384	1:8192	1:32768
B	1:1024	1:512	1:256	1:1024

To determine the limits of experimental error for the test for antihyaluronidase, four sera, and normal human serum gamma globulin³ were used as controls and were tested along with patients' sera. The results of these titrations on different days are tabulated in Table V. The greatest variation in antihyaluronidase titre of any of the control sera was a two-tube (or four-fold) difference. These results would seem to establish the limits of experimental error for the test sera as two two-

³ Prepared by the Department of Physical Chemistry, Harvard Medical School, in cooperation with the Antitoxin and Vaccine Laboratory, Mass. Dept. of Public Health, from blood collected by American Red Cross for the Committee on Medical Research, Office of Scientific Research and Development.

TABLE V

Serial tests for antihyaluronidase of four control samples of serum and serum gamma globulin

Date of test	Control serum enzyme titre				
	Frick	Aldrich	Raymond	Faracles	Serum gamma globulin
12/26/46	1:65,536				
3/18/47			1:1024		
3/26/47			1:1024		
3/21/47			1:1024		
			1:1024		
3/31/47		1:1024			
4/ 7/47	1:16,384	1:1024		1:1024	1:32,768
4/12/47	1:16,384	1:4096		1:1024	1:32,768
4/14/47	1:16,384	1:2048		1:1024	1:32,768
4/16/47		1:2048		1:512	1:32,768
4/17/47		1:2048			
4/21/47		1:2048		1:512	1:16,384
4/23/47					1:16,384
4/29/47	1:32,768	1:4096			
	1:65,536	1:4096			
5/ 2/47	1:65,536	1:1024			
5/ 7/47		1:1024			
5/ 8/47	1:32,768				
5/ 9/47	1:16,384	1:1024			
5/15/47	1:65,536	1:4096			
5/19/47	1:32,768	1:2048			
5/23/47	1:32,768	1:1024			
	1:32,768	1:1024			
5/24/47			1:2048		
5/26/47	1:16,384	1:1024			
5/27/47	1:32,768	1:1024			
7/ 4/47	1:32,768	1:1024			

fold dilutions. It is important to make serum dilutions with fresh serum each day because if sera are previously diluted and stored in a refrigerator, the antienzyme titre decreases markedly within a few days.

In order to determine the effect of different strengths of hyaluronidase on the antihyaluronidase titre of sera, 14 sera were tested against different enzyme strengths. Early in the study, sera were tested against 64 or 128 units of enzyme. Later, all sera were tested against 16 units of enzyme. Serial two-fold dilutions of serum were tested against 128, 64, 32, 16, 8 and 4 units of enzyme, respectively. The results of some of these titrations are plotted in Figure 3. In general a linear relationship was observed when antienzyme titre was plotted against enzyme units. If a linear relationship were always observed, theoretically a given serum dilution would inhibit 128 units of enzyme, a two-fold greater dilution of serum would inhibit 64 units of enzyme, and so on. But such a relationship was not always observed, particularly in sera with very high antienzyme titres. In

sera with lower antienzyme titres the linear relationship held, particularly in the range between 64 to 16 units of enzyme. The antihyaluronidase titres of sera tested against 64 or 128 units of enzyme were extrapolated in terms of 16 units of enzyme. Four of the sera as shown in Figure 3 (M.M., R.L., L. and D.A.) had antihyaluronidase titres which fell along a perfect straight line when plotted against units of enzyme within the range between 64 to 16 units. In only two instances (F. and D.A.) did the observed antihyaluronidase titre differ more than a two-fold dilution from the predicted antihyaluronidase titre.

Employing the (M.C.P.) method for testing

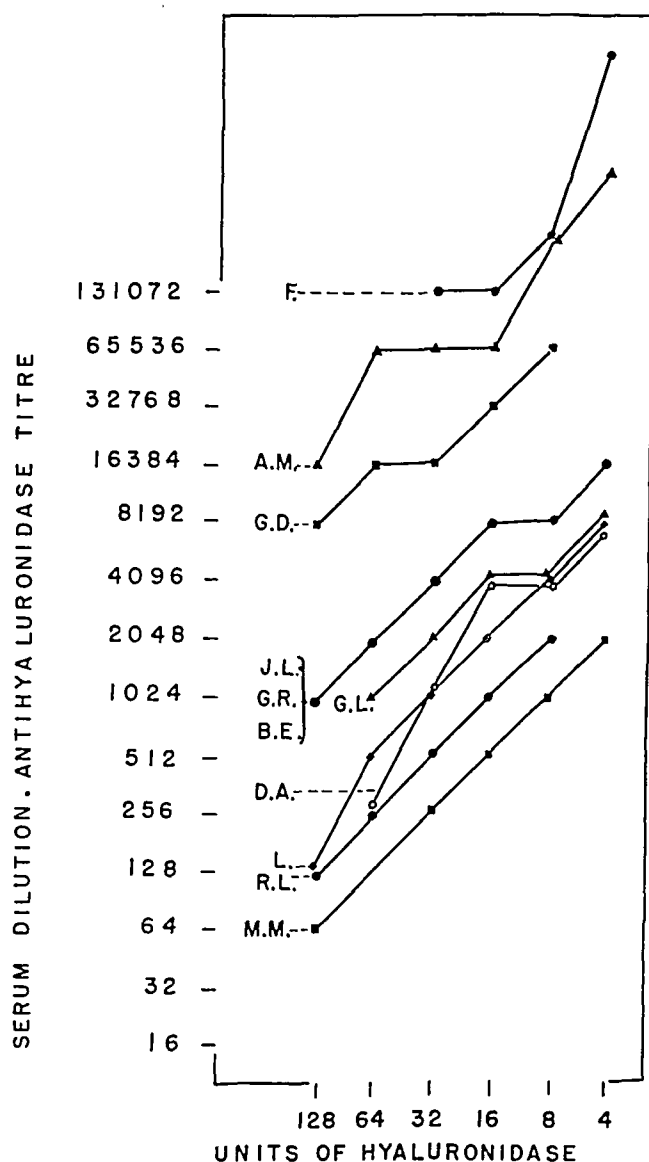


FIG. 3. RESULTS OF TESTS OF SERIAL TWO-FOLD DILUTIONS OF SERUM AGAINST SERIAL TWO-FOLD DILUTIONS OF *STREPTOCOCCUS* GROUP A, TYPE 4 HYALURONIDASE

antihyaluronidase, 16 sera from ten individuals were tested against hyaluronidase obtained from a strain of Group A, type 4 hemolytic streptococcus, a strain of staphylococcus aureus, and against purified bull's testicle hyaluronidase.⁴ The sera all showed the presence of an inhibitory substance against hyaluronidase from the hemolytic streptococcus, little or no antienzyme against the staphylococcus enzyme and no inhibition of the testicle enzyme. These results are similar to those reported by Friou and Wenner (1). Furthermore, using the viscosity reducing method described by Haas (30) eight of the sera exhibited the presence of an inhibitory substance against enzyme from bull's testicle (Schering).⁵ These same sera contained no antienzyme against staphylococcus or bull's testicle (Schering) enzyme when tested according to the mucin-clot prevention test of McClean *et al.* (32) but they did exhibit antienzyme against streptococcus enzyme in considerable amounts. Neither normal calf serum nor normal horse serum contained any antienzyme against hyaluronidase from any of the above sources when tested with the mucin-clot prevention test.

Twenty-four sera in all including normal horse serum and normal calf serum were tested by the viscosity reducing method and all were found to contain a substance which would inhibit the hydrolysis of hyaluronic acid by hyaluronidase from bull's testicle. This substance has been termed antinvasin I by Haas (30). The substance which will prevent the action of hyaluronidase in the mucin-clot prevention test has been termed antihyaluronidase.

DISCUSSION

Setting up the mucin-clot prevention test for antihyaluronidase so that the serum being tested rather than the enzyme is the variable factor, has increased the efficiency of the test as a measure of antihyaluronidase activity. Previously when the enzyme was the variable factor, some sera would inhibit undiluted enzyme thereby making an end point unobtainable. The method described in

this study will always enable one to obtain an end point of antihyaluronidase titre. Two-fold serum dilutions rather than three-fold enzyme dilutions increase the accuracy of the test. It is believed that the accuracy of the test can be increased further by using standardized preparations of hyaluronidase and potassium hyaluronate.

The linear relationship which was observed after plotting the results of testing sera diluted serially in two-fold decrements against serial dilutions of beta hemolytic streptococcal enzyme in two-fold decrements seems to make it possible to extrapolate the antihyaluronidase titres of sera tested against 64 or 128 units of enzyme to the titre which would be predicted if the sera were tested against 16 units of enzyme.

When a few sera were tested by means of the viscosity reducing method they exhibited the property of inhibiting the viscosity reducing action of hyaluronidase from bull's testicle (Schering). However, when the same sera were tested by the mucin-clot prevention test the property of inhibition of clot production by hyaluronidase from bull's testicle (Schering) was not present nor was there any appreciable inhibition of hyaluronidase from staphylococci. These sera did, however, exhibit significant inhibition of hyaluronidase from a strain of Group A, type 4 hemolytic streptococcus. The reasons for these discrepancies are not clear but from the work of McClean (2) it would appear that viscosimetry and the mucin-clot prevention test measure the activity of the same agent but that destruction of the clotting power of the substrate occurs earlier than any appreciable fall in viscosity. Possibly a longer incubation period of serum-enzyme mixtures will lessen the differences between the two methods in measuring antihyaluronidase activity. Studies are in progress in an effort to clarify the significance of these differences.

SUMMARY AND CONCLUSIONS

1. Slight modifications of methods for the determination of the hyaluronidase activity of certain strains of hemolytic streptococci and staphylococci as well as hyaluronidase extracted from testes have been described.

2. A modification in the method of determining the antihyaluronidase titre of sera by means of the mucin-clot prevention test whereby the serum

⁴ The purified bull's testicle hyaluronidase was furnished through the courtesy of Dr. Erwin Schwenk of Schering Corporation, Bloomfield, N. J.

⁵ This method was not used routinely because it is so much more time-consuming than the M.C.P. test.

rather than the enzyme is the variable factor were made. The limits of error of this method at present is about two two-fold dilutions, but it is believed that the accuracy can be increased.

3. Serial two-fold dilutions of serum tested against serial two-fold dilutions of streptococcal hyaluronidase, in general, exhibited a definite relationship; i.e.: if a serum dilution of 1:512 would inhibit 128 units of enzyme, the same serum diluted to 1:1024 would inhibit 64 units of enzyme, etc. This linear relationship was most constant when sera were tested against hyaluronidase strength within the range between 64 to 16 units.

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ANTIHYALURONIDASE STUDIES OF SERA FROM PATIENTS WITH RHEUMATIC FEVER, STREPTOCOCCAL INFECTIONS, AND MISCELLANEOUS NON-STREPTOCOCCAL DISEASES¹

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INTRODUCTION

In the previous paper the technical aspects of the mucin-clot prevention test which was standardized by McClean (1) have been described. Friou and Wenner (2) using the mucin-clot prevention test have demonstrated the clinical significance of an inhibitory substance in human serum capable of neutralizing an enzyme elaborated by a strain of hemolytic streptococcus. The amount of inhibitory substance was shown by them to be greater in the sera of patients with rheumatic fever than in patients early in the course of uncomplicated hemolytic streptococcal infections or normal individuals. A brief historical background of the work preceding that of Friou and Wenner has been presented in the previous paper.

The purpose of this paper is to report the clinical application of the mucin-clot prevention test in the determination of the antihyaluronidase titre of sera of patients with rheumatic fever, hemolytic streptococcal infections, miscellaneous other diseases, and normal individuals.

MATERIALS AND METHODS

The materials and methods used in these studies were exactly as described in the previous paper.

Enzyme: The enzyme used throughout in these tests was that produced by a strain of Group A, type 4 beta hemolytic streptococcus. Each serum was tested against a constant amount (16 units) of hyaluronidase.

Substrate: The substrate was composed of a 0.15% solution of potassium hyaluronate in distilled water, normal horse serum in a dilution of 1:10 in physiological saline, and distilled water in a ratio of 1:1:2, respectively. Potassium hyaluronate was prepared according to the method of McClean *et al.* (3).

Serum: The blood used in the study of normal individuals and patients was drawn as aseptically as possible.

The serum was separated from the clot within 24 hours in most instances and stored in lusteroid tubes at -70°C .³

Methods of determining the mean antihyaluronidase titre:

An arbitrary code was devised and numbers were assigned to each titre, *e.g.*:

Serum dilution	Code
0	— .2
1:16	1
1:32	2
1:64	3
etc.	4

An equation for determining the code was devised.⁴

$$\text{Code} = \frac{\log \text{reciprocal titre} - .9013}{- .3010}$$

and

$$\log \text{reciprocal titre} = (- .3010 \times \text{code}) - .9031$$

Example: For a serum with the antihyaluronidase titre 1:4096 the code would be:

$$\begin{aligned} \text{Code} &= \frac{\log 4096 - .9031}{- .3010} \\ &= \frac{3.6124 - .9031}{- .3010} \\ &= \frac{-2.7093}{- .3010} = 9 \end{aligned}$$

The mean antihyaluronidase titre for a group of sera was determined by the formula below:

$$\frac{\text{codes of sera}}{\text{number of sera}} = \text{mean code.}$$

The titre to which the code number was arbitrarily assigned was then determined.

³ Blood specimens were collected from the wards of the New Haven Hospital and Dispensary Clinic. They also were obtained from patients at the U. S. Naval Hospital at St. Albans, Long Island, through the courtesy of Capt. H. L. Weaver and Capt. W. D. Small, from patients in the St. Francis Sanatorium for Cardiac Children at Roselyn, L. I., through the courtesy of Dr. Leo Taran and the Rev. Mother Superior, and from patients in the Children's Center, New Haven, Conn., through the assistance of Dr. C. W. Wooltruff.

⁴ Dr. John H. Watkins, Assoc. Prof. of Public Health devised this formula and his advice was followed in determining the statistical significance of the results.

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² Milbank Memorial Fund Fellow 1946-47.

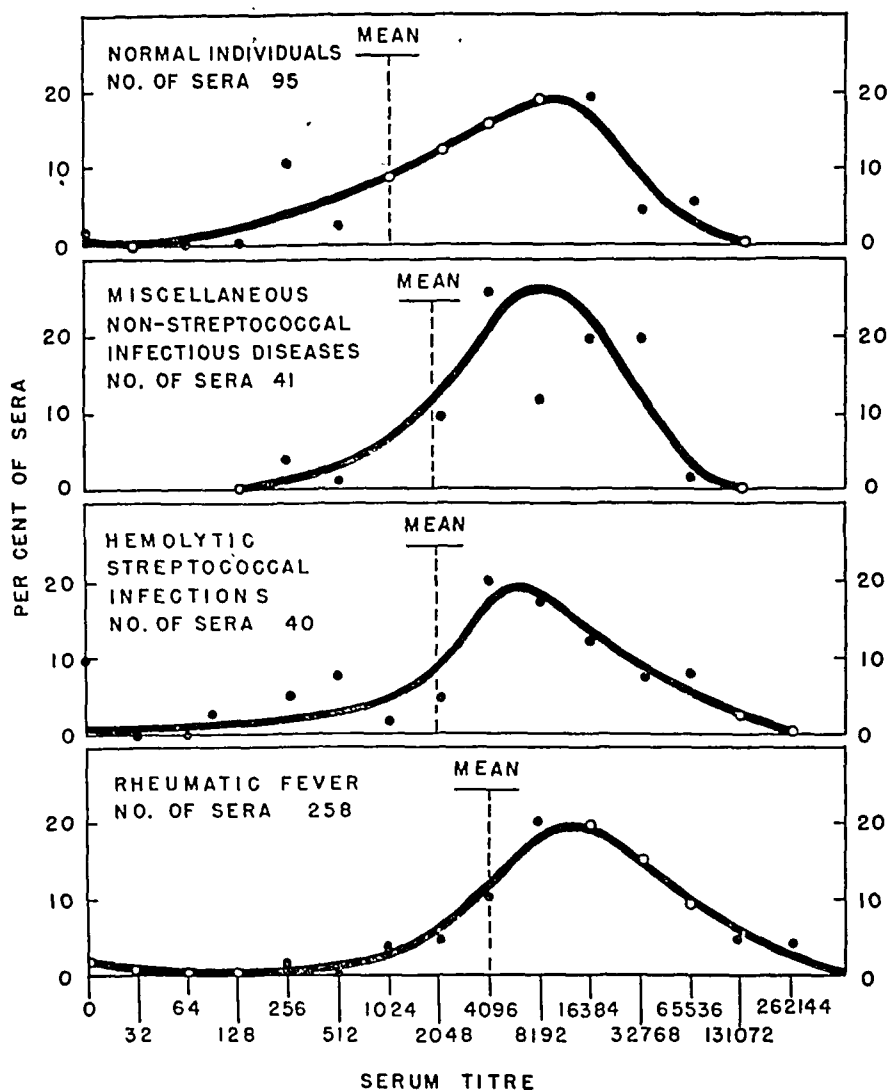


FIG. 1. FREQUENCY DISTRIBUTION OF ANTIHYALURONIDASE TITRES FOR SERA FROM PATIENTS WITH RHEUMATIC FEVER, HEMOLYTIC STREPTOCOCCAL INFECTIONS, MISCELLANEOUS NON-STREPTOCOCCAL INFECTIOUS DISEASES, AND NORMAL INDIVIDUALS

RESULTS

A total of 495 sera from 387 individuals was tested. As a base line the mean antihyaluronidase titre of 95 normal adult sera was first determined as 1:1024. The mean antihyaluronidase titre of all groups of sera are recorded in Table I and Figure 1. Subsequently the mean of 40 sera from patients convalescent from scarlet fever and other acute beta hemolytic streptococcal infections was determined to be slightly less than 1:2048. That of ten patients with active rheumatoid arthritis was 1:1024. Forty-one sera from patients with non-streptococcal infectious diseases including tuberculosis, syphilis, bacterial and "virus" pneumonia, leprosy, Vincent's angina, gonococcus urethritis,

etc. had a mean titre slightly less than 1:2048. The mean antihyaluronidase titre for 41 sera from the patients with non-streptococcal infectious diseases combined with the ten sera from patients with rheumatoid arthritis was slightly less than 1:2048. Sera from all the 258 patients with rheumatic fever had a mean antihyaluronidase titre of 1:4096. The sera from patients with rheumatic fever were analyzed further according to the state of activity of the rheumatic process at the time the blood was collected. The mean antihyaluronidase titres for these different groups of sera were as follows: For rheumatic fever, active, acute, it was higher than 1:16,384 being slightly nearer to 1:16,384 than the next highest dilution which would be 1:32,768. In this group of patients

TABLE I

Mean antihyaluronidase titre of sera from groups of patients according to diagnosis

Diagnosis	Number of sera	Mean antihyaluronidase titre
Normal Individuals	95	1:1024
Miscellaneous Non-streptococcal Infectious Diseases	41	slightly less than 1:2048
Miscellaneous Non-streptococcal Infectious Diseases plus Rheumatoid Arthritis	51	slightly less than 1:2048
Rheumatoid Arthritis, Active	10	slightly higher than 1:1024
Scarlet Fever plus other Beta Hemolytic Streptococcal Infections	40	slightly less than 1:2048
Rheumatic Fever	258	1:4096
	495	
Rheumatic Fever, Active, Acute	15	higher than 1:16,384
Rheumatic Fever, Active	83	midway between 1:4096 and 1:8192
Rheumatic Fever, Active, Subsiding	31	slightly higher than 1:8192
Rheumatic Fever, Active, Chronic	21	midway between 1:2048 and 1:4096
Rheumatic Fever, Inactive	81	slightly higher than 1:2048

were those whose illness had begun within three weeks of the time the serum was collected and who still had high fever, acute arthritis, tachycardia, carditis, and other clinical and laboratory evidence of acute rheumatic fever. These patients were all receiving salicylates by mouth in therapeutic doses. The highest individual antihyaluronidase titres were observed in this group of patients. The titre of some sera in this group was as high as 1:262,144 and furthermore this high titre was not observed in sera from any individuals other than those with rheumatic fever, active, acute. The mean titre for the group with rheumatic fever, active, *i.e.*, those whose illness was more than three weeks old but who still had evidence of ac-

tivity as manifested by fever, tachycardia, carditis, rapid sedimentation rate, electrocardiographic changes, etc., was midway between 1:4096 and 1:8192. The group with active but subsiding rheumatic fever had a mean antienzyme titre slightly higher than 1:8192. About one fourth of the patients with active, or active, subsiding rheumatic fever were receiving salicylates. The mean titre of the sera from patients whose illness was classified as rheumatic fever, active, chronic, was midway between 1:2048 and 1:4096. These patients had been ill with low-grade, active rheumatic fever for many months. Two were receiving salicylates. The mean antihyaluronidase titre for sera from patients with inactive rheumatic fever was slightly higher than 1:2048.

In the statistical analysis of the differences between the mean antihyaluronidase titres of groups of sera, the *t* test (4) was employed. Values of *t* of 1.96 or greater were interpreted to mean that the differences between the mean titres of the two groups of sera under comparison were statistically significant.

The mean antihyaluronidase titre of sera from rheumatic fever patients of 1:4096 was significantly higher than the mean titre of sera from any other group of patients or from normal individuals. These comparisons are recorded in Table II along with the computed standard difference, the standard error of difference and the value of *t*. The frequency distribution of the antihyaluronidase titres of sera from each group of patients is plotted in Figure 1. This figure shows graphically the

TABLE II

Statistical analysis of the differences between the mean titres of groups of sera

Number of sera being compared		Standard difference	Standard error of difference	<i>t</i>	Statistically significant
Rheumatic Fever (258)	vs. Normal Individuals (95)	2.135	0.256	8.04	Yes
Rheumatic Fever (258)	vs. Scarlet Fever and other Beta Hemolytic Streptococcal Infections (40)	2.330	0.396	5.95	Yes
Rheumatic Fever (258)	vs. Miscellaneous Diseases plus Rheumatoid Arthritis (51)	2.042	0.343	4.19	Yes
Scarlet Fever plus other Streptococcal Infections (40)	vs. Miscellaneous Diseases plus Rheumatoid Arthritis (51)	2.773	0.552	1.59	No
Miscellaneous Diseases plus Rheumatoid Arthritis (51)	vs. Normal Individuals (95)	2.609	0.492	0.803	No
Rheumatic Fever, Active, Acute (15)	vs. Normal Individuals (95)	2.093	0.363	1.94	No
Rheumatic Fever, Active, Acute (15)	vs. Normal Individuals (95)	3.088	0.8579	5.042	Yes
Rheumatic Fever, Active, Acute (15)	vs. Miscellaneous Non-streptococcal Diseases (41)	0.2849	0.8597	3.99*	Yes
Rheumatic Fever, Active, Acute (15)	vs. Scarlet Fever and other Beta Hemolytic Streptococcal Infections (40)	2.436	0.7377	6.264	Yes
Rheumatic Fever, Active, Acute (15)	vs. Rheumatic Fever, Active (83)	2.626	0.736	2.35	Yes
Rheumatic Fever, Active, Acute (15)	vs. Rheumatic Fever, Active, Subsiding (31)	2.1659	0.6512	1.67	No
Rheumatic Fever, Active, Acute (15)	vs. Rheumatic Fever, Active, Chronic (21)	1.911	0.2042	13.429	Yes
Rheumatic Fever, Active, Acute (15)	vs. Rheumatic Fever, Inactive (81)	1.941	0.5454	5.423	Yes

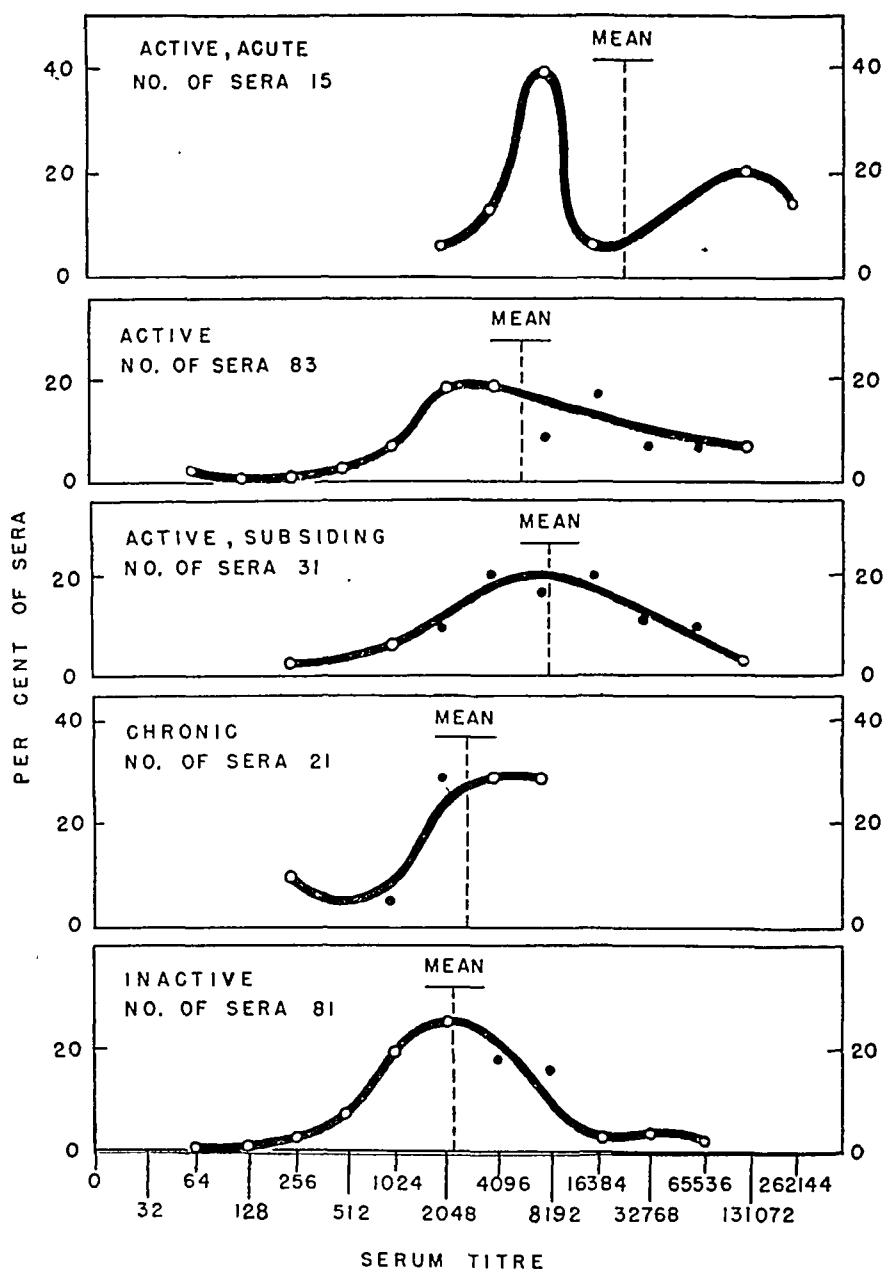


FIG. 2. FREQUENCY DISTRIBUTION OF ANTIHYALURONIDASE TITRES OF SERA FROM PATIENTS WITH RHEUMATIC FEVER IN DIFFERENT STAGES OF ACTIVITY

wider range of titres and higher mean titre of the rheumatic fever sera compared with sera from patients with hemolytic streptococcal infections, non-streptococcal infectious diseases, and normal individuals.

One of the important results of these studies was the finding that the mean antihyaluronidase titre of the sera from patients with rheumatic fever, active, acute, was significantly higher than the mean titre of sera from normal individuals or from any other group of patients except those with rheumatic fever, active, subsiding. The mean titre of

sera from patients with rheumatic fever, active, acute, was actually higher than the mean titre from patients with rheumatic fever active, subsiding, but the difference was not statistically significant. The mean titre of sera from patients with rheumatic fever, active, acute, however, was significantly higher than the mean titre of the sera from patients in any other phase of rheumatic fever. The frequency distribution of antihyaluronidase titres of sera from different groups of rheumatic fever patients was plotted in Table II. Here is shown the higher range of titres of sera from pa-

tients with rheumatic fever, active, acute, and the gradual decrease in mean antihyaluronidase titre from the group, with rheumatic fever, active, acute, to the group with rheumatic fever, inactive.

DISCUSSION

In these studies the contention of Friou and Wenner (2) that the amount of inhibitory substance against streptococcal hyaluronidase in sera from patients with rheumatic fever was greater than in sera from patients early in the course of uncomplicated hemolytic streptococcal infections or from normal individuals has been confirmed. It also has been demonstrated that the serum antihyaluronidase titre in patients early in the course of active rheumatic fever is significantly higher than the antihyaluronidase titres of sera from any other group of patients studied including patients with active, subsiding, or inactive rheumatic fever, hemolytic streptococcal disease, and non-streptococcal infectious diseases. The results do not yield information which indicates that patients with streptococcal disease have a higher mean antihyaluronidase titre than patients with other infectious diseases. However, further studies of the antihyaluronidase titre in patients during the course of streptococcal infection and rheumatic fever along with the antifibrinolysin and antistreptolysin "O" titres are being done and will be reported later.

No attempt has been made to study the effect of salicylates on the antihyaluronidase titre of patients or the effect of salicylates on hyaluronidase *in vivo* and *in vitro*, but from the recent reports by Guerra (5), Pike (6), Dorfman *et al.* (7), and Meyer (8), it would appear that salicylates do inhibit the spreading effect of hyaluronidase in skin but have no inhibitory effect on testicular or bacterial hyaluronidase *in vivo* in concentrations obtained therapeutically.

Obviously much more investigation is necessary

before the meaning of these results will be fully understood or before this test can be proposed as a diagnostic measure.

SUMMARY AND CONCLUSIONS

1. Studies of the determination of antihyaluronidase titres of sera from patients with rheumatic fever, hemolytic streptococcal infections, miscellaneous non-streptococcal infectious diseases, and normal individuals have been presented.

2. The mean antihyaluronidase titre of sera from patients with rheumatic fever was significantly higher than the mean titre of sera from the other groups of patients studied.

3. The mean antihyaluronidase titre of sera from patients with rheumatic fever, active, acute, was significantly higher than the mean antihyaluronidase titre of sera from patients with rheumatic fever in less active forms and from sera from patients in other groups studied.

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THE NITROUS OXIDE METHOD FOR THE QUANTITATIVE DETERMINATION OF CEREBRAL BLOOD FLOW IN MAN: THEORY, PROCEDURE AND NORMAL VALUES¹

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In 1945 the authors reported the determination of cerebral blood flow in man by the use of nitrous oxide in low concentration, a technique which permitted for the first time quantitative clinical measurement of this important physiologic function (1). Since that time numerous modifications have been made in the procedure (2) and the underlying theory has been subjected to extensive experimental evaluation. The present report constitutes a description of the technique as we have now employed it in over 300 determinations, an examination of its underlying theory and validity, and values obtained with its use in 34 studies on 14 normal young men.

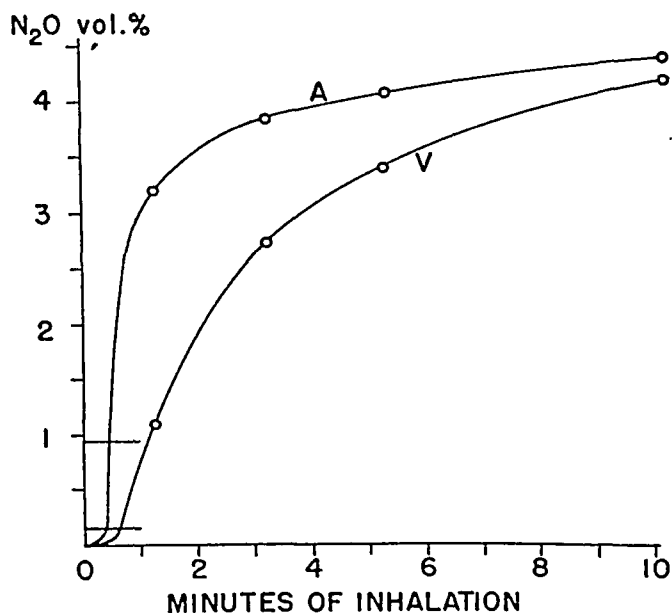


FIG. 1. TYPICAL ARTERIAL (A) AND INTERNAL JUGULAR (V) CURVES OF N₂O CONCENTRATION DURING A TEN-MINUTE PERIOD OF INHALATION OF 15 PER CENT N₂O

¹ The expenses of these investigations were defrayed by grants from the Office of Scientific Research and Development and the Committee on Research in Dementia Praecox founded by the Supreme Council, 33rd Scottish Rite, Northern Masonic Jurisdiction, U. S. A.

THEORY

If the nitrous oxide concentration be determined in arterial and cerebral venous blood for a ten-minute period from the beginning of the inhalation of a low concentration of this gas, curves will be obtained similar to the typical ones in Figure 1. The venous concentration curve is a fairly complex function of the arterial curve and the cerebral blood flow (3) but from these curves the cerebral blood flow can be calculated by the application of the familiar Fick principle. This postulates in its simplest form that the quantity of any substance taken up in a given time by an organ from the blood which perfuses it is equal to the total amount of the substance carried to the organ by the arterial inflow less the amount removed by the venous drainage during the same time period. For the case of the brain uptake of N₂O:

Let $(Q_B)_u$ = quantity of N₂O taken up by the whole brain in time u measured from the start of inhalation,

$(Q_A)_u$ = quantity brought to the brain by the arterial blood in time u ,

$(Q_V)_u$ = quantity carried away by cerebral venous blood in time u ,

A = arterial N₂O concentration,

V = venous N₂O concentration,

W = weight of brain,

TF = total cerebral blood flow per minute,

CBF = cerebral blood flow per unit weight of brain per minute.

From the Fick principle:

$$(Q_B)_u = (Q_A)_u - (Q_V)_u$$

but, since both A and V are variables with respect to time,

$$(Q_A)_u = TF \int_0^u A dt$$

and

$$(Q_V)_u = TF \int_0^u V dt,$$

whence

$$(Q_B)_u = TF \int_0^u (A - V) dt$$

or

$$TF = \frac{(Q_B)_u}{\int_0^u (A - V) dt} \quad (1)^2$$

² This is a general formula for the Fick principle applying to any organ or the whole body and to any substance. In the special case of its use for determination of cardiac output using O₂ or CO₂ the denominator

or, in terms of unit weight of brain,

$$CBF = \frac{(Q_B)_u/W}{\int_0^u (A-V)dt}, \quad (2)$$

The denominator is readily obtained from the respective arterial and cerebral venous curves.³ The numerator, or the cerebral concentration of nitrous oxide, is not obtainable directly in man. If the time u is sufficiently long, however, equilibrium will have occurred between brain and the blood leaving the brain with respect to nitrous oxide tension. At that time:

$$\frac{(Q_B)_u}{W} = V_u S, \quad (3)$$

where S represents a partition coefficient for nitrous oxide between brain and blood. By substituting appropriately and multiplying through by 100, one obtains a value for cerebral blood flow in convenient units:

$$CBF = \frac{100 V_u \cdot S}{\int_0^u (A-V)dt}, \quad (4)$$

where CBF is expressed as cc. of blood flow per 100 g. of brain per minute.

DISCUSSION

Before this technique can be regarded as capable of yielding valid measurements of cerebral blood flow the assumptions on which it is based must be subjected to experimental verification. This was recognized in our original description of the method (1) and evidence was adduced to validate the assumptions. Since that time it has been possible to make a more exhaustive evaluation of these assumptions and to determine more precisely the value of the constant S .

Blood from one internal jugular bulb as representative of mixed cerebral venous blood

The method yields values for mean cerebral blood flow only insofar as the venous blood samples obtained from one internal jugular are representative of mixed cerebral venous blood. Three studies of oxygen content in samples taken simultaneously or in rapid succession from both right

and left internal jugular have revealed significant differences between the two sides in as many as one third of the subjects (4 to 6). Himwich and associates (7) recently reported what they interpreted as significant differences between the two sides with respect to cerebral blood flow and oxygen consumption determined by the nitrous oxide method in a number of patients. They conclude that one internal jugular is predominantly representative of cortical drainage while the other largely drains the basal ganglia, and find anatomical justification for this conclusion in the statement that "except in the small proportion of human subjects who have torculars, the two internal jugular veins do not drain symmetrical portions of the brain." Some doubt arises both as to the validity of this statement and the conclusion based upon it. According to the results of Edwards (8), Manno (9), and Riggs (10) who studied a total of 125 specimens, there is a real confluence of the sinuses in approximately two thirds of the population. But even in that minority of patients where a confluence does not exist or is markedly lopsided one cannot neglect the fact that the torcular is only one of many means whereby mixing may occur. According to Cobb (11), the venules which emerge from the cortex are continuous with those which pass through the subcortical regions to drain into the internal venous system; there is thus no clear-cut differentiation of cortical from subcortical blood even at their origin. An examination of the larger veins of the brain (12) reveals many opportunities for mixing exclusive of the torcular. Thus in a patient where the superior sagittal sinus drained almost completely to the right lateral sinus and the straight sinus to the left, the left internal jugular would also receive "cortical" blood from the entire left inferior surface of the cerebrum, from the anastomotic veins of Trolard and L'abbe and from the left half of the cerebellum, while the right internal jugular would receive in addition to blood from the superior sagittal sinus, "subcortical" blood from the cavernous sinus, the cerebellum, pons and medulla. In other words, from the smaller architectural units through the major dural sinuses there is little evidence for segregation of cortical venous outflow from the drainage of subcortical tissue.

In an effort to obtain a more definitive answer

becomes simply $(A-V)t$ since the arteriovenous differences of these gases are presumed to be constant.

³ In our original report the integral was approximated by a single exponential function. Although this was fairly accurate there is no need for fitting the arteriovenous difference into any rigid formula, since whatever its nature its integral can be found graphically or by the trapezoid rule.

TABLE I
An evaluation of the anatomical and experimental errors of the method

Simultaneous measurements on right and left internal jugular blood									Successive measurements on the same side								
Patient	(V-A)CO ₂		(A-V)O ₂		Cerebral				Patient	(V-A)CO ₂		(A-V)O ₂		Cerebral			
					Blood flow		O ₂ consumption							Blood flow		O ₂ consumption	
	Right	Left	Right	Left	Right	Left	Right	Left		I	II	I	II	I	II	I	II
	vol. %	vol. %	vol. %	vol. %	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.		vol. %	vol. %	vol. %	vol. %	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.
E. B.	6.1	6.7	6.1	6.4	45	42	2.7	2.7	J. Mc.	6.2	7.1	7.0	6.8	48	42	3.4	2.9
S. S.	6.7	6.7	5.4	5.6	64	64	3.5	3.6	V. Z.	5.3	6.3	5.5	6.1	50	46	2.8	2.8
*M. M.	1.3	1.3	2.0	2.3	149	136	3.0	3.1	R. B.	5.9	6.1	6.7	7.0	57	57	3.8	4.0
U. P.	10.6	11.7	8.0	8.2	50	53	4.0	4.3	W. R.	5.1	6.1	5.3	6.0	59	57	3.1	3.4
W. R.	5.6	5.3	5.7	5.5	46	56	2.6	3.1	J. Sk.	5.1	5.0	5.5	5.6	57	60	3.1	3.4
J. S.	5.0	5.3	5.9	5.7	61	75	3.6	4.3	G. A.	5.1	5.2	5.1	5.8	62	55	3.2	3.2
W. L.	6.5	6.4	6.8	6.6	58	53	3.9	3.5	U. P.	7.2	6.1	8.3	5.9	36	50	3.0	3.0
R. B.	6.1	5.8	6.4	6.2	65	57	4.2	3.5	W. L.	6.1	6.6	6.0	6.1	61	56	3.7	3.4
*F. B.	1.1	2.4	1.7	2.1	201	169	3.4	3.6									
M. T.	6.9	6.5	7.2	7.1	48	47	3.5	3.3									
Mean	5.59	5.81	5.52	5.57	78.7	75.2	3.44	3.50	Mean	5.75	6.06	6.18	6.16	53.8	52.9	3.26	3.26
Standard deviation of the differences within an individual							±0.281		Standard deviation of the differences within an individual							±0.187	

* These two patients had large unilateral cerebral hemangiomas.

to this question we made measurements of cerebral blood flow by means of the nitrous oxide technique, and of arteriovenous oxygen and carbon dioxide differences, simultaneously on both internal jugular bulbs in a series of ten patients. These included two patients with unilateral cerebral hemangiomas, the resultant arteriovenous shunt producing a tremendous increase in cerebral blood flow locally and straining to its utmost the mixing capacity of the cerebral venous drainage. The results are presented in Table I. The agreement between bilateral measurements in any one patient is quite good, the standard deviation of the individual differences for cerebral oxygen consumption is ± 0.28 cc. O₂/100 g./min. This includes not only the anatomical variation in drainage but also all the sampling and analytical errors inherent in the estimations of CBF and (A-V)O₂. These errors of the method may be estimated from the set of duplicate determinations on the same side in another series of patients before and after a procedure which produced no variance *per se* (13). Here the standard deviation of the differences between duplicates was ± 0.19 cc. O₂/100 g./min., which is not significantly less than 0.28. Thus the differences found between the right and left in-

ternal jugular measurements are within the experimental error of the method, which is also quite small. It would be essential to know similarly the experimental error of the nitrous oxide method as employed by Himwich and associates (7) in evaluating the differences which they found between the two sides.

The extent of contamination of internal jugular blood at the level of the superior bulb, with blood of extracerebral origin

Evidence that one internal jugular contains blood which is adequately representative of both does not exclude the possibility that both may be equally contaminated with blood which arises outside the brain. Examination of the cerebral venous drainage (12) reveals several emissary veins or communications between the cerebral and extracerebral drainage systems. Although their size in comparison with the total of cerebral veins would indicate that such contamination could hardly be important, it was nevertheless necessary to arrive at a measurement of the fraction of internal jugular blood at its superior bulb having extracerebral origin. This has been done in a series of eight patients in whom the carotids on one side were ex-

posed in preparation for cerebral angiography (12). In these patients a dye (T-1824) could be injected into an external carotid artery while samples were slowly taken from the internal jugular bulbs and the external jugular vein. By comparing the dye concentration in the internal jugular with that in the external jugular, it was possible to arrive at a fairly quantitative measure of the extent of this contamination.⁴ It averaged 2.6% with a maximum value of 6.5%. It is interesting to note that when the procedure was reversed and the dye injected into the internal carotid, significant amounts appeared in the external jugular indicating that on the average about 20% of external jugular blood is of cerebral origin. The older anatomists who named these communications *emissary* veins anticipated these results. A possible source of significant contamination is the common facial vein which joins the internal jugular several centimeters below the superior bulb. For this reason it is important that the needle be placed high in the superior bulb and that blood samples be taken from this needle at a rate slow enough to insure against the possibility of drawing blood in a retrograde direction from the lower parts of the vein.

From the results of these two studies, necessarily limited in number by the obvious technical difficulties involved, it is possible to conclude that in the great majority of individuals blood from one internal jugular at the level of the superior bulb is fairly representative of mixed cerebral venous blood not significantly contaminated with blood from extracerebral sources. Exceptions may occur, but are unlikely to constitute a significant fraction of the population. This is also borne out by the comparatively small spread of our data on cerebral oxygen consumption, in a series of 34 observations on normals (Table III) and 30 studies on schizophrenics (13). Although the possibility of gross anatomical variation in any individual case still remains, it is not great and does not compromise the validity of results obtained or conclusions drawn from a statistically significant series of cases.

⁴ This would not include, however, that part of the venous return from the eye supplied by the internal carotid and draining into the cavernous sinus.

Equilibration between brain and cerebral venous blood

Although it is obvious that there must be a time during the inhalation of a constant tension of inert gas when the brain is in equilibrium with the blood leaving it with respect to this gas, this time interval must be evaluated. In our original report we presented indirect evidence that after ten minutes practical equilibrium between brain and cerebral venous blood had been achieved. Since that time we have been able to make direct analyses of the nitrous oxide contents of brain and cerebral venous blood in dogs exposed to nitrous oxide for varying times (14). These studies have demonstrated that ten minutes is sufficient for the attainment of equilibrium between brain and cerebral venous blood with respect to nitrous oxide tension. The value for u in Equation 4 may therefore be taken as ten minutes.

The partition coefficient (S) of N₂O between brain and blood

The same experiments yield a value for S which is very close to unity (0.98). These *in vivo* results compare very well with the partition coefficients obtained *in vitro* for dog (1.03) and human (1.06) brains (14).⁵

Although the nitrous oxide curves are capable of yielding a value for flow per unit N₂O capacity:

$$CBF/S = \frac{V_u}{\int_0^u (A-V)dt},$$

regardless of the absolute value of S , flow in terms of unit weight of brain is dependent on the constancy of the partition coefficient among different individuals and in the same individual at different

⁵ On the basis of these more accurate studies it appears that our previous tentative value of 1.3 for this factor was in error. This value had been derived from N₂O curves on monkeys obtained simultaneously with direct measurement of cerebral blood flow using the bubble-transfer flowmeter (1). Re-examination of these experiments revealed the source of error: the length of time which the arterial blood spent in the meter before reaching the brain. This rendered the samples taken from the artery and internal jugular vein not really simultaneous. Recalculation of these curves to correct this error yields a mean value for the factor S of 1.07, more comparable with that obtained by the other methods.

TABLE II

Comparison between the nitrous oxide method and simultaneous direct measurement of CBF

Rhesus or Spider Monkeys

Experiment	Bubble meter flow	N ₂ O flow
	cc./100 g./min.	cc./100 g./min.
24	37	31
27	42	33
28	17	20
30 I	46	37
30 II	60	54
30 III	31	34
31 I	38	34
31 II	76	71
31 III	32	37

times. This coefficient, however, depending on gross physico-chemical constitution would be expected to change significantly only with such changes in brain or blood composition as would be incompatible with life (14).

Final corroboration of the validity of the nitrous oxide method is to be found in comparison between it and the direct flow measurement by

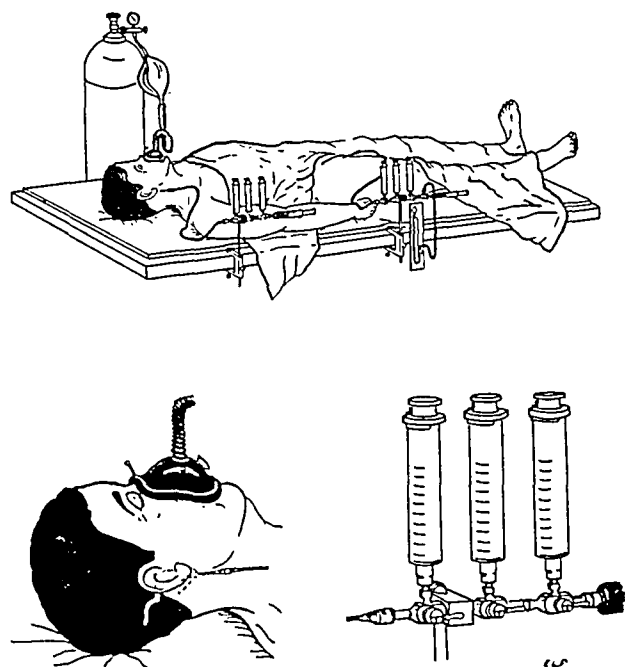


FIG. 2. EXPERIMENTAL SET-UP FOR CEREBRAL BLOOD FLOW DETERMINATION

Position of needles in internal jugular is shown as well as plastic tubing, manifolds, sampling and flushing syringes, and inhalation system. Mean arterial blood pressure is read from a mercury manometer attached to the arterial manifold. Only the expiratory valve on the mask is shown, the inspiratory valve is between the fluted tubing and the mask.

(Drawings by Dr. E. L. Foltz)

means of the bubble flowmeter performed simultaneously in monkeys (Table II). These experiments have been reported previously (1); now, however, the systematic error due to the interposition of the bubble meter has been removed. The nitrous oxide technique employed had not been refined to its present state, but the recently determined value of 1 for the partition coefficient has been used in the calculations. It is seen that the agreement between the two methods is quite satisfactory.

METHODS

Blood sampling

For taking the accurately timed serial blood samples from artery and internal jugular vein, manifolds of three-way stopcocks⁶ (Figure 2) have been found very convenient (2). The syringes (10-cc. Luer-Lok) are prepared beforehand by lightly oiling the plunger with mineral oil, filling the dead space with heparin solution and sealing with the closed hubs of discarded hypodermic needles. Transparent plastic tubing of small bore⁷ connects the manifold to the needle (19 G 3" spinal) through suitable adaptors. The needle is placed in the superior bulb of the internal jugular after procaine infiltration according to the technique of Myerson, Halloran and Hirsch (15) as recently modified by Gibbs, Lennox and Gibbs (4). We have found this technique readily tolerated and very dependable; there have been only two failures in the last 200 attempts. For arterial blood we have used the femoral or brachial arteries. After the needle is in place it is carefully connected to the plastic tubing which has been filled with sterile heparin solution (10 mg./cc.) by means of the end syringe containing 3 cc. of heparin (Figure 2). Just before each sample is taken 3 cc. of blood are drawn into this syringe to clear the system and at the conclusion of each sample 3 cc. of the blood-heparin mixture in this syringe are pushed back to prevent clotting.

The following samples of 6-8 cc. each are taken: X, a blank taken from the vein just before the beginning of N₂O inhalation; 1A and 1V taken at a constant rate (1 cc. every ten sec.) from the onset of N₂O inhalation over the first minute; 2A and 2V from 1'5" to 1'30"; 3A and 3V from 2'45" to 3'15"; 4A and 4V from 4'45" to 5'15"; 5A and 5V from 9'45" to 10'15". Of course the actual times are a matter of indifference so long as they are accurately noted and fairly evenly spaced. Samples are sealed and iced until analysis. The N₂O mixture consists of 15% N₂O, 21% O₂, and 64% N₂, kept in large cylinders under pressure.

The gas is administered in an open system through an anesthesia bag and tightly fitting mask equipped with

⁶ These were constructed for us by Mr. D. W. T. Cochrane of this laboratory.

⁷ "Transflex," 14 gauge, made by Irvington Insulator Co., Irvington, N. J.

inspiratory and expiratory valves. It is very important that these valves be competent and the mask fit perfectly. Unless the patient breathes a constant tension of N_2O throughout the flow the arterial and venous curves will not be smooth functions and cannot accurately be drawn from only five pairs of samples.

It is possible to perform a second measurement on the same patient while the needles are still in place. It is important when this is done that at least 20 minutes elapse between CBF determinations for cerebral N_2O desaturation to occur and of course a blank blood sample is taken just before the second determination.

Analysis of blood samples for N_2O

Because of the number of analyses necessary for each blood flow determination it has been necessary to modify the original method of Orcutt and Waters (16) making it simpler, more accurate and more rapid (2): Two drops of caprylic alcohol and 9 cc. of distilled water are de-aerated in the Van Slyke-Neill manometric apparatus, then run up to the 5 cc. mark of the cup. Two cc. of blood, shaken in the syringe by means of a droplet of mercury and transferred directly to an Ostwald-Van Slyke pipette, are run into the chamber and washed in with 1 cc. of de-aerated water. The cup is cleared with gentle suction and 3 cc. of the usual alkaline O_2 absorber (sodium hyposulfite, sodium anthraquinone-beta-sulfonate, potassium hydroxide), previously de-aerated and stored anaerobically, are added to the cup and the lower 2 cc. run into the chamber. No air bubbles should be present in the chamber at this time. The chamber is sealed with mercury and the mixture extracted for three minutes, then allowed to rise smoothly to the 2 cc. mark. Readings are made of pressure (r_a) and temperature to 0.1 mm. and 0.1 degree. The mixture is now expelled and the next analysis begun without washing the chamber. A single blank analysis using 2 cc. of de-aerated water instead of blood yields a reading which, after correction for difference in water vapor tension resulting from a temperature change between the blank and each analysis, may be used as the r_0 for each analysis. A blood blank (X) is also necessary to correct for other gases (largely N_2) or N_2O remaining from a previous flow.

$$\text{vol. \% } N_2O = f'_{N_2O}(r_a - r_0) - X, \\ X = f'_{N_2O}(r_x - r_0).$$

The manometric factor (f'_{N_2O}) for 15% N_2O and 64% N_2 varies linearly from a value of 0.1456 at 20° C. to 0.1383 at 30° C. The procedure above corrects not only for nitrogen but also for its inverse relationship to nitrous oxide during the period of gas administration.⁸ Under these circumstances the value for X should be close to 1.15 vol. % unless a previous flow measurement has been performed. If 20 minutes is permitted to elapse between successive flows, nitrous oxide desaturation will be nearly

complete and X will be found to be only 0.1 or 0.2 vol. % higher than 1.15. With ordinary care duplicate analyses for blood N_2O should agree within 0.05 vol. %. After sufficient skill has been acquired it is possible to forego duplicate analyses, using the smoothness of the resultant curves as a check on the individual analyses.⁹

Calculation of cerebral blood flow

When the nitrous oxide analyses are completed the values are plotted against time. The time of each sample is taken as the mid-time of the interval over which the sample was taken except for the first pair, taken at a constant rate over the first minute (and therefore already integrated) which are plotted as lines. Smooth curves are then drawn through the arterial and venous points and so constructed over the first minute that the average samples obtained ($1\bar{A}$ and $1\bar{V}$) approximate the respective integrals of the curves (Figure 1). From these smooth curves values for A , V , and $A-V$, are recorded at the end of each minute over the ten-minute period. $\int_0^1 (A-V) dt$ is obtained directly as the difference between the first pair of samples (taken at a constant rate and hence automatically integrated); the remaining integrals are calculated by means of the trapezoid rule, i.e., $\int_1^2 (A-V) dt = \frac{(A-V)_1 + (A-V)_2}{2}$; $\int_2^3 (A-V) dt = \frac{(A-V)_2 + (A-V)_3}{2}$, etc. It is then possible by serial addition to calculate values of $\int_0^u (A-V) dt$ and thence $\frac{100V_u}{\int_0^u (A-V) dt}$ for serial values of u from one to ten minutes (2). The latter function, in a typical study, decreases rapidly over the first five or six minutes but in the last several minutes tends to level off. This tendency serves as an internal check on the final result since equilibration between brain and venous blood should be complete inside of ten minutes.¹⁰ In most cases this function is not perfectly constant at that time indicating the small amount of contamination from extracerebral sources. Occasionally one obtains a study in which this function is falling rapidly even at ten minutes; this is evidence that contamination is significant and the study should be discarded. We have observed this only twice in our last 100 studies. Since the partition coefficient (S) is unity and the equilibration time is ten minutes, the value of this function at $u = \text{ten minutes}$ represents the cerebral blood flow expressed as cc. per 100 g. of brain/min.

⁹ A modification is necessary in the Van Slyke-Neill manometric technique for CO_2 and O_2 to prevent an error due to the solubility of nitrous oxide in the respective absorbing reagents: These are added at the appropriate times but instead of a slow addition this may be made quite rapidly, after which the upper cock is sealed with mercury, the mixture extracted at the 50 cc. mark for two minutes, raised smoothly and read at the 2 cc. volume.

¹⁰ For this reason we prefer to make the calculation as described rather than calculate the entire ten-minute integral at once. The additional work involved is negligible.

⁸ Since the solubility of N_2O in blood is more than 32 times that of N_2 the error due to nitrogen even if uncorrected is small; with proper correction it vanishes.

With the value for cerebral blood flow it is now possible to arrive at a measurement of some extremely important functions. The utilization or production by the brain of any substance capable of accurate analysis in arterial and cerebral venous blood is estimated quantitatively by substitution in the transposed Fick formula. Thus for the cerebral utilization of oxygen (CMR_{O_2}):

$$CMR_{O_2} \text{ (cc. } O_2/100 \text{ g. brain/min.)} = CBF \times \frac{(A-V)_{O_2}}{100}$$

if $(A-V)_{O_2}$ is expressed as vol. %. A value for cerebrovascular resistance (CVR) is calculable from the mean carotid blood pressure, the internal jugular pressure, and CBF :

$$CVR = \frac{(\text{mean carotid BP} - \text{mean jugular BP}) \text{ mm. Hg.}}{CBF \text{ (cc./100 g./min.)}}$$

CVR is obtained in convenient units representing the pressure necessary to force 1 cc. of blood per minute through 100 g. of brain. This may be converted to absolute units by means of an appropriate factor. In practice a satisfactory approximation is derived using the femoral arterial mean pressure for the carotid pressure and neglecting the jugular venous pressure.

NORMAL VALUES

In the course of our studies on the effects of altered carbon dioxide and oxygen tensions on cerebral blood flow (17, 18) we have obtained a series of 34 measurements of cerebral blood flow and cerebral oxygen consumption in addition to several other observations in 14 healthy young men lying at rest. Mean values and standard deviations are given in Table III. These subjects

TABLE III

*Blood flow and oxygen consumption of the human brain
(34 observations on 14 healthy young men)*

	Mean	σ^*
Cerebral blood flow (cc./100 g./min.)	54	± 12
Cerebral O_2 consumption (cc./100 g./min.)	3.3	± 0.4
Cerebrovascular resistance (mm.Hg/cc. blood/ 100 g. brain/min.)	1.6	± 0.4
Cerebral arteriovenous O_2 difference (vol. %)	6.3	± 1.2
Cerebral respiratory quotient	0.99	± 0.09
Mean femoral arterial B.P. (mm. Hg)	86	± 7

* σ refers to the deviation among individuals.

were all cooperative, well motivated and intelligent; disturbing factors such as fear and apprehensiveness were at a minimum. Although they do not represent a complete cross section of the population these data constitute the only quantitative study yet reported of blood flow and metabolism in the normal human brain.

These values for cerebral oxygen consumption are comparable to values found in the rhesus

monkey by a more direct method (19), although the human values are about 15% lower than those for the monkey. The ever present but slight factor of contamination by the extracerebral circulation would work in opposite directions in the two methods used, possibly causing the nitrous oxide method to underestimate the true value by several per cent and the bubble flowmeter to overestimate the figure to a like degree. This factor alone would explain the small discrepancy although species differences would certainly be expected to exist.

SUMMARY

1. The nitrous oxide method for measurement of cerebral blood flow in unanesthetized man is described and its errors estimated.
2. The fundamental assumptions on which the method is based have been subjected to experimental verification.
3. Comparison between the nitrous oxide technique and direct measurement of flow by means of the bubble flowmeter in monkeys shows excellent agreement between the two methods.
4. In a group of normal young men the mean value for cerebral blood flow was 54 ($\sigma = \pm 12$) cc./100 g./min. The mean value for cerebral oxygen consumption was 3.3 ($\sigma = \pm 0.4$) cc. O_2 /100 g./min.

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THE EFFECTS OF ALTERED ARTERIAL TENSIONS OF CARBON DIOXIDE AND OXYGEN ON CEREBRAL BLOOD FLOW AND CEREBRAL OXYGEN CONSUMPTION OF NORMAL YOUNG MEN¹

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A method for measuring quantitatively the volume of cerebral blood flow in man by inhalation of nitrous oxide (1) found its first application in a study of the cerebral circulatory effects of low CO₂ tension achieved by hyperventilation; of high CO₂ tension, and of high and low O₂ tensions obtained by inhalation of appropriate gas mixtures (2). Only the first part of this study, the effects of active and passive hyperventilation, has been published in detail (3). The purpose of the present paper is to present the remainder of these findings and to derive from them, together with those of the hyperventilation experiments, evidence bearing on the intrinsic control of the human cerebral circulation as revealed by quantitative measurements.

METHODS

The nitrous oxide technique is described in a preceding report (4). The subjects were young male volunteers in apparently good health. A set of control observations were made after the fasting subject had rested supine for more than an hour. After the control period, the experimental gas mixture (free of nitrous oxide) was administered for 15 to 30 minutes in order to approximate a steady state, and for the removal of N₂O absorbed by the brain during the control cerebral blood flow determination. At the end of this time a change was quickly made to a gas mixture similar to the preceding one but containing 15% N₂O and a second or "experimental" series of observations was made. The composition of the gas mixtures used was as follows: (1) for hyperventilation, room air followed by 21% O₂, 64% N₂, 15% N₂O; (2) for increased CO₂, 5 or 7% CO₂, 21% O₂, 74 or 72% N₂ followed by 5 or 7% CO₂, 21% O₂, 59 or 57% N₂, 15% N₂O; (3) for high O₂, 100% O₂ followed by 85% O₂, 15% N₂O; (4) for low O₂, 10% O₂, 90% N₂ followed by 10% O₂, 75% N₂, 15% N₂O.

Blood O₂ and CO₂ analyses were made by the manometric technique of Van Slyke and Neill (5). Blood

pH measurements were made anaerobically at 37° C. using a glass electrode. CO₂ tension was calculated by means of the nomograms of Peters and Van Slyke (5). Mean arterial blood pressure was obtained directly from the femoral artery by means of a damped mercury manometer; systolic and diastolic pressures were also measured by the usual auscultatory method. In the middle of both the control and experimental periods ballistocardiograms were recorded. These were used for calculation of a value for cardiac output from the formula of Starr and associates (6), using the correction factor of 1.18 found by Courmand, Ranges and Riley (7).

Cerebral oxygen consumption (CMR_{O₂}) and cerebrovascular resistance (CVR) were calculated from the cerebral blood flow as described previously (4).

RESULTS

The essential data obtained are presented in Tables I, II and III. Mean values obtained with active or passive hyperventilation and previously reported (3) are included for the sake of completeness, with a correction in the cerebral blood flow and oxygen consumption values for the more accurate determination of the brain: blood partition coefficient of N₂O (1.0 instead of the previously used value of 1.3). The arterial and internal jugular N₂O concentration curves from which the cerebral blood flow is calculated are omitted for the sake of brevity but typical curves are shown in Figure 1.

Effects of CO₂ inhalation (5-7%)

There were six studies with 5% CO₂ and two with 7% CO₂; these were grouped together for calculation of mean values. Arterial blood CO₂ content, CO₂ tension, and hydrogen ion concentration all rose as expected. Cerebral blood flow underwent a striking and consistent increase averaging 75% (from a mean control value of 53 to a mean value of 93 cc./100 g./min.). Cerebral oxygen consumption was not significantly changed with the result that the increased volume flow was

¹The expenses of these investigations were defrayed by grants from the Office of Scientific Research and Development and the Committee on Research in Dementia Precox, founded by the Supreme Council, 33° Scottish Rite, Northern Masonic Jurisdiction, U. S. A.

associated with an equivalent decrease in the arteriovenous oxygen difference. Along with the increased cerebral blood flow there was a marked reduction in the mean cerebrovascular resistance from 1.6 to 1.1 resistance units. With respect to the general circulatory effects of CO₂, there was a significant rise in arterial blood pressure though cardiac output was not significantly altered. This speaks for a net peripheral vasoconstriction, an effect quite opposite to that exerted on the cerebral circulation.

Effects of high oxygen concentrations

Inhalation of the high oxygen mixtures (85–100%) produced a slight but significant increase

in arterial oxygen content with no change in the CO₂ content and tension or the pH of arterial blood. This speaks against any appreciable effects of these oxygen tensions on pulmonary ventilation at least during the first 30 minutes of inhalation. There was a significant decrease of 13% in mean cerebral blood flow (from 52 to 45 cc./100g./min.) with no change in cerebral oxygen consumption. Cerebrovascular resistance exhibited a moderate increase (from 1.7 to 2.2 resistance units) indicating vasoconstriction in the brain as the probable mechanism for the reduction in cerebral blood flow. There was a significant increase in systolic and diastolic blood pressure on auscultatory measurement and in mean arterial pressure measured di-

TABLE I
Effects of altered arterial CO₂ and O₂ tensions on blood constituents

Gas		Subject	Blood CO ₂ content				Blood CO ₂ tension				Blood O ₂ content				Blood pH			
			Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular	
			C†	E‡	C	E	C	E	C	E	C	E	C	E	C	E	C	E
21% O ₂	5% CO ₂	S. H.	46.7	49.9	54.0	54.3	42	50	55	58	16.0	16.1	9.5	11.4	7.38	7.33	7.30	7.28
		T. L.	49.4	51.7	55.7	57.2	42	47	53	54	15.7	15.0	9.6	10.7	7.38	7.36	7.33	7.33
		R. Ro.	47.3	50.7	55.1	55.0	41	48	53	56	16.5	16.6	9.6	11.4	7.38	7.34	7.32	7.30
		W. S.	51.5	53.2	57.1	57.4	48	54	60	60	16.5	16.8	10.7	12.7	7.35	7.31	7.29	7.28
		D. M.	48.3	50.4	54.6	55.1	41	46	50	51	18.6	18.5	12.6	14.0	7.40	7.36	7.35	7.33
	R. S.	48.5	52.0	53.8	54.8	42	53	54	58	14.8	15.5	9.8	12.3	7.37	7.30	7.30	7.27	
	7% CO ₂	J. B.	51.7	55.6	57.7	58.6	45	60	58	66	18.2	18.4	11.9	16.0	7.38	7.28	7.31	7.26
		W. H.	52.5	56.9	58.8	59.5	45	58	52	59	18.3	18.7	12.1	16.0	7.40	7.32	7.35	7.31
		Mean	49.5	52.5*	55.9	56.5*	43	52*	54	58*	16.8	16.9	10.7	13.0*	7.38	7.33*	7.32	7.30
	85-100% O ₂		J. E.	48.2	47.9	55.3	55.7	39	37	48	48	17.8	19.1	10.8	11.1	7.42	7.45	7.38
T. L.			50.1	50.8	58.0	59.1	41	40	51	56	15.5	16.7	7.6	8.3	7.41	7.42	7.36	7.34
S. H.			51.0	49.3	55.8	55.1	43	41	51	50	16.5	17.6	16.6	12.2	7.39	7.40	7.33	7.34
R. Re.			51.1	50.9	56.7	57.4	42	42	52	53	16.1	17.5	10.8	10.9	7.40	7.40	7.34	7.34
J. B.			49.3	49.5	55.6	56.7	43	43	53	54	16.2	17.8	10.5	11.2	7.38	7.39	7.32	7.32
M. H.			51.4	51.6	57.5	58.7	42	42	52	53	17.2	19.0	11.2	11.5	7.41	7.41	7.36	7.35
Mean			50.2	50.0	56.5	57.1	42	41	51	52	16.6	18.0*	10.4	10.9*	7.40	7.41	7.35	7.35
10% O ₂			R. S.	47.7	48.2	52.4	50.9	41	38	49	43	16.1	9.7	11.1	6.4	7.38	7.41	7.33
	S. H.		50.6	50.9	55.5	53.9	41	37	48	43	17.0	11.0	11.8	7.0	7.42	7.46	7.37	7.41
	K. T.		45.4	44.9	53.0	48.9	35	31	46	33	17.8	11.6	10.8	7.8	7.45	7.50	7.38	7.68
	W. H.		50.7	49.7	57.1	54.6	42	36	51	44	18.7	13.1	12.4	7.6	7.41	7.48	7.36	7.41
	D. M.		46.8	45.9	54.5	51.5	40	34	51	42	19.4	13.2	12.5	8.0	7.40	7.47	7.34	7.41
	M. H.		49.1	49.0	56.2	53.4	42	37	51	45	17.7	11.2	10.7	6.5	7.39	7.43	7.34	7.40
	J. E.		47.0	46.6	54.5	51.5	39	36	51	43	19.0	12.3	10.8	7.5	7.40	7.45	7.34	7.38
	Mean		48.2	47.9	54.7	52.1*	40	36*	50	41	18.0	11.7*	11.4	7.3*	7.41	7.46*	7.35	7.41*
Hyperventilation active and passive Mean			50.1	41.7*	55.9	52.5*	45	26*	52	38*	17.9	18.6*	11.3	7.8*	7.38	7.54*	7.35	7.48*

* Indicates statistically significant changes.

† C = Control period, room air tensions of O₂ and CO₂.

‡ E = Experimental period, special gas mixture.

TABLE II
Effects of altered arterial CO₂ and O₂ tensions on cerebral circulation and metabolism

Inspired gas		Subject	Cerebral									
			Blood flow		O ₂ consumption		Vascular resistance		RQ		A-VO ₂	
			C†	E‡	C	E	C	E	C	E	C	E
21% O ₂	5% CO ₂	S. H.	cc./100 g./min.		cc./100 g./min.		mm. Hg cc./100 g./min.				vol. %	
		T. L.	48	65	3.1	3.1	1.7	1.4	1.12	.94	6.5	4.7
		R. Ro.	50	67	3.1	2.9	1.6	1.3	1.03	1.28	6.1	4.3
		W. S.	46	75	3.2	3.9	1.8	1.2	1.13	.83	6.9	5.2
		D. M.	63	90	3.7	3.7	1.3	0.9	.97	1.02	5.8	4.1
		R. S.	56	80	3.4	3.6	1.4	1.2	1.05	1.05	6.0	4.5
	7% CO ₂	J. B.	63	141	3.2	3.9	1.3	0.7	1.06	1.00	5.0	2.8
		W. H.	53	135	3.3	3.2	1.7	0.8	.95	1.25	6.3	2.4
		Mean	45	90	2.8	2.4	1.9	1.0	1.01	1.00	6.2	2.7
85-100% O ₂	J. E.	40	34	2.8	2.7	2.2	2.7	1.01	.98	7.0	8.0	
	T. L.	50	52	4.0	4.4	1.8	1.9	1.00	.99	7.9	8.4	
	S. H.	60	49	2.9	2.6	1.4	1.8	.98	1.07	4.9	5.4	
	R. Re.	61	55	3.2	3.7	1.5	2.1	.96	.98	5.3	6.7	
	J. B.	57	41	3.2	2.7	1.5	2.3	1.10	1.09	5.7	6.6	
	M. H.	43	39	2.6	2.9	2.0	2.5	1.02	.95	6.0	7.5	
	Mean	52	45*	3.1	3.2	1.7	2.2*	1.01	1.01	6.1	7.1*	
10% O ₂	R. S.	71	93	3.6	3.1	1.2	0.9	.94	.82	5.0	3.3	
	S. H.	60	81	3.1	3.2	1.2	0.8	.96	.75	5.2	4.0	
	K. T.	44	82	3.1	3.1	2.0	1.0	1.08	1.05	7.0	3.8	
	W. H.	47	58	3.0	3.2	1.9	1.2	1.01	.90	6.3	5.5	
	D. M.	57	67	3.9	3.5	1.5	1.2	1.11	1.08	6.9	5.2	
	M. H.	52	75	3.6	3.5	1.8	1.0	1.01	.94	7.0	4.7	
	J. E.	44	54	3.6	2.6	2.1	1.6	.92	1.02	8.2	4.8	
	Mean	54	73*	3.4	3.2	1.7	1.1*	1.00	.94	6.6	4.5*	
Hyperventilation active and passive												
Mean		52	34*	3.5	3.7	1.7	2.9*	0.88	1.00	6.6	10.8*	

* Indicates statistically significant changes.

† C = Control period, room air tensions of O₂ and CO₂.

‡ E = Experimental period, special gas mixture.

rectly, but there were no observable changes in cardiac rate, stroke volume, or minute output. Here also there must have been vasoconstriction accompanying the inhalation of these concentrations of oxygen, but in this case the cerebral vessels participated.

Effects of 10% oxygen

The relative anoxia produced by inhalation of 10% oxygen was reflected in a pronounced fall in arterial oxygen content from a control value of 18.0 to 11.7 vol. %. Although there were no measurements of pulmonary ventilation an increase in this function must certainly have oc-

curred to account for the observed significant decreases in pCO₂ and hydrogen ion concentration found in arterial blood. Cerebral blood flow was regularly increased, the average rising from 54 to 73 cc./100g./min., an increase of 35%, which occurred in the face of a significant reduction in femoral mean arterial pressure. There was a decrease in cerebrovascular resistance from 1.7 to 1.1, showing that anoxia of this degree was just as effective as 5-7% CO₂ in dilating cerebral vessels even though cerebral blood flow was considerably more augmented in the latter case because of the contributing effects on the systemic circulation. There was no consistent or significant change in

TABLE III
Effects of altered CO₂ and O₂ tensions on circulatory functions

Inspired gas	Subject	Stroke volume ml				Cardiac output liters/min.				Pulse rate				Auscultatory blood pressure mm. Hg				Mean arterial blood pressure direct mm. Hg	
		CI†	CH	CHH	E‡	CI	CH	CHH	E	CI	CH	CHH	E	CI	CH	CHH	E	CHH	E
21% O ₂	S. H.	76	74	72	63	4.1	3.9	3.4	3.9	54	53	47	62	98/68	96/62	100/68	112/80	82	94
	T. L.	64	67	67	63	4.0	3.9	3.7	3.5	62	58	55	55	106/70	112/75	106/68	115/76	78	84
	R. R.	63	66	60	63	2.8	4.0	3.6	3.5	45	60	60	55		98/65	98/70	108/68	84	89
	W. S.	76	73	73	74	6.0	6.1	5.8	6.3	79	83	78	85	100/60	100/58	108/62	110/62	80	83
7% CO ₂	D. M.	80	92	92	79	5.2	5.4	5.9	5.8	65	59	64	74	105/68	105/70	110/70	120/82	81	96
	R. S.	73	76	72	81	4.5	4.9	4.3	5.7	62	65	60	71		110/68	118/72	125/78	80	93
	J. B.		70	77	66		4.3	4.3	4.5	62	61	65	65	104/70	104/70	105/72	128/85	88	110
	W. H.	77	81	75	80	5.2	5.9	5.6	5.7	68	73	74	72	100/66		110/72	110/80	86	93
	Mean	73	75	74	72	4.5	4.8	4.7	5.1	62	64	62	69	102/66	104/67	107/69	116*/76*	82	93*
85-100% O ₂	J. E.		86	88	83	5.6	4.9	5.7	5.4	63	57	65	65		95/63	100/78	105/78	89	93
	T. L.	89	67	59	65		4.0	3.4	3.8		60	57	59		112/82	108/70	115/85	88	100
	S. H.		75	71	68		5.0	4.0	3.8		66	57	56		105/70	100/70	115/80	83	86
	R. R.		86	90	85		3.9	4.1	4.0		45	45	47		115/70	120/70	135/98	89	114
10% O ₂	J. B.		76	80	75		3.9	4.2	4.0		51	52	53		105/75	105/70	115/85	84	96
	M. H.		65	68	66		3.9	4.3	3.5		60	64	53		105/75	112/72	120/80	88	96
	Mean		76	76	73		4.3	4.3	4.1		57	57	56		106/73	108/72	118*/84*	87	98*
	R. S.		74	76	72		4.7	4.5	6.6		64	59	92		106/76	105/75	112/70	87	86
Hyperventilation active and passive Mean	S. H.		79	78	78		4.9	4.4	5.5		62	56	71		102/75	102/70	90/62	75	64
	K. T.		83	79	75		3.7	3.5	5.9		45	44	78		108/76	108/76	108/65	87	79
	W. H.		77	76	77		5.4	5.2	5.2		70	69	68		105/85	108/76	102/62	88	71
	D. M.		76	87	86		5.6	5.9	5.8		74	68	68		104/70	104/70	94/68	83	79
	M. H.		60	60	54		3.2	4.9	4.9		48	54	90		100/63	122/82	122/82	93	76
	J. E.		83	83	78		4.8	5.6	6.6		58	68	84		115/85	114/88	108/65	91	86
	Mean		79	77	74*		4.9	4.6	5.8*		60	60	79*		106/76	109/76	105/68*	86	78*
			72	69	60*		5.0	4.7	4.5		70	68	75		109/77		112/86	90	98*

* Indicates statistically significant changes.

† CI, CH, CHH = Control periods at approximately 30 minute intervals.

‡ E = Experimental period.

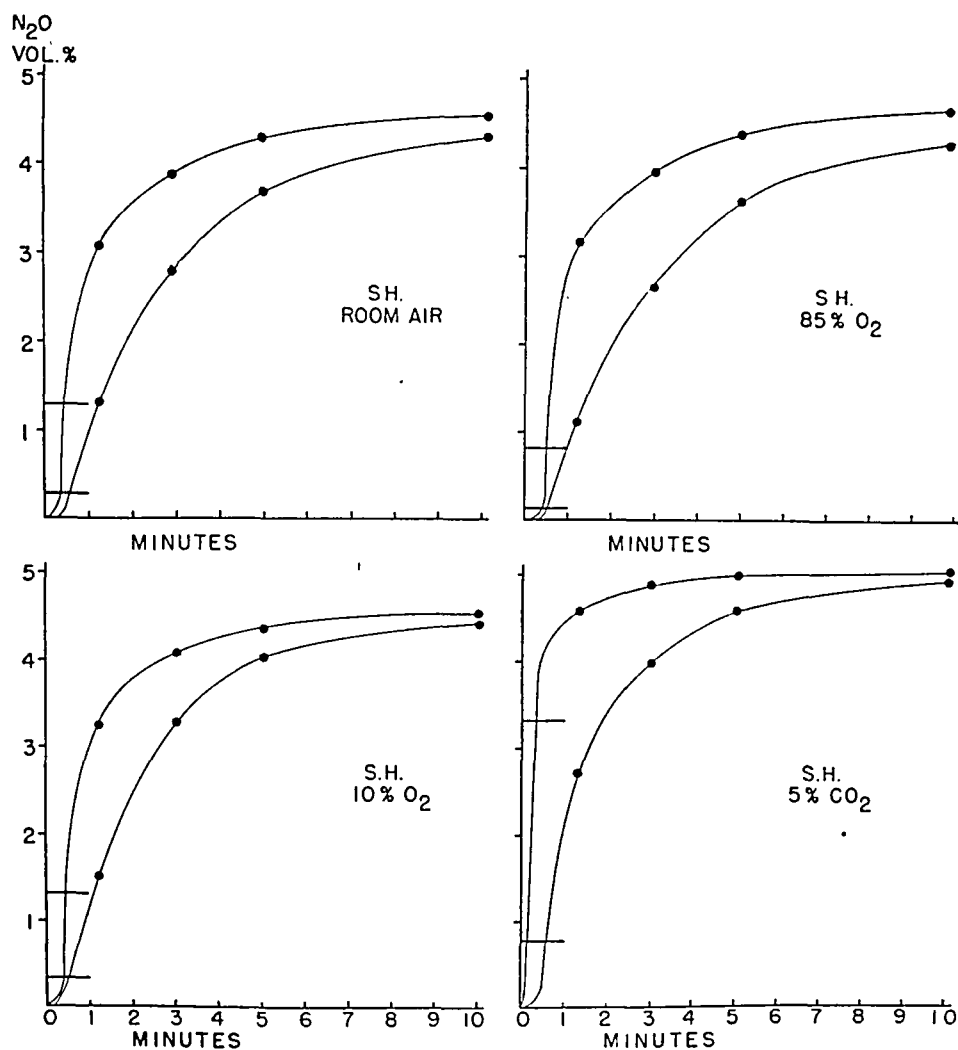


FIG. 1. SAMPLE N₂O CURVES OBTAINED ON ONE SUBJECT AT DIFFERENT TIMES REPRESENTING ONE OF THE ROOM AIR CONTROL PERIODS, 85% O₂, 10% O₂ AND 5% CO₂.

cerebral oxygen consumption. The general circulatory effects of anoxia in this group were a significant increase in cardiac output resulting from an acceleration in ventricular rate, yet a fall in mean arterial blood pressure, suggesting a considerable degree of peripheral vasodilatation.

DISCUSSION

These experimental findings lend the support of quantitative measurements in man to the already prevalent belief (8 to 11) that the cerebral blood vessels are strongly influenced by the carbon dioxide and oxygen tensions of the arterial blood. They also afford a previously unavailable insight into some important relationships here involved, for the reason that they permit quantitative comparisons in the normal, intact state. Previous observations in unanesthetized man were made by

methods which did not justify quantitative deductions (9, 10, 12) and the only truly quantitative measurements in animals were made under conditions more or less remote from the normal (13).

It is of interest to compare these results on cerebral blood flow with studies on the coronary circulation, the most recent being those on the heart *in situ* of Eckenhoff, Hafkenschiel and Landmesser (14) who found a reduction of 11% with pure oxygen, a 64% increase with 10% oxygen, but little effect from inhalation of 5-7% carbon dioxide.

The data on cerebral oxygen consumption demonstrate that except for an increase associated only with active hyperventilation (3) there is no significant change in oxygen utilization by the brain in the ranges of gas tension studied. This lends substance to the assumption made by investigators

who used the cerebral arteriovenous oxygen difference as a measure of blood flow under similar circumstances (9), although it is difficult to see a justification for such an assumption *a priori*. Indeed, it is somewhat surprising that neither hyperventilation nor anoxia showed any depression of cerebral oxygen utilization, even though both were accompanied by definite mental changes. These were the only cases, however, which exhibited any decrease in internal jugular oxygen content and this was markedly reduced in both (7.3 and 7.8 vol. % for 10% O_2 and hyperventilation, respectively). The possibility presents itself that a reduced mean cortical pO_2 , as reflected in the lower-

ing of the pO_2 of internal jugular blood, might well be a factor in producing the mental effects. Thus derangements in consciousness may occur when the complex oxidation processes with which consciousness is associated are forced to operate at a lowered oxygen tension even though the gross oxygen consumption by the whole brain may be within normal limits. The conclusion is apparent that the higher psychic functions are associated with biochemical changes so subtle and complex as to render any attempt to describe them in terms of mere oxygen utilization no more adequate than to predict the fidelity of a radio by its power requirements. This is not to imply the con-

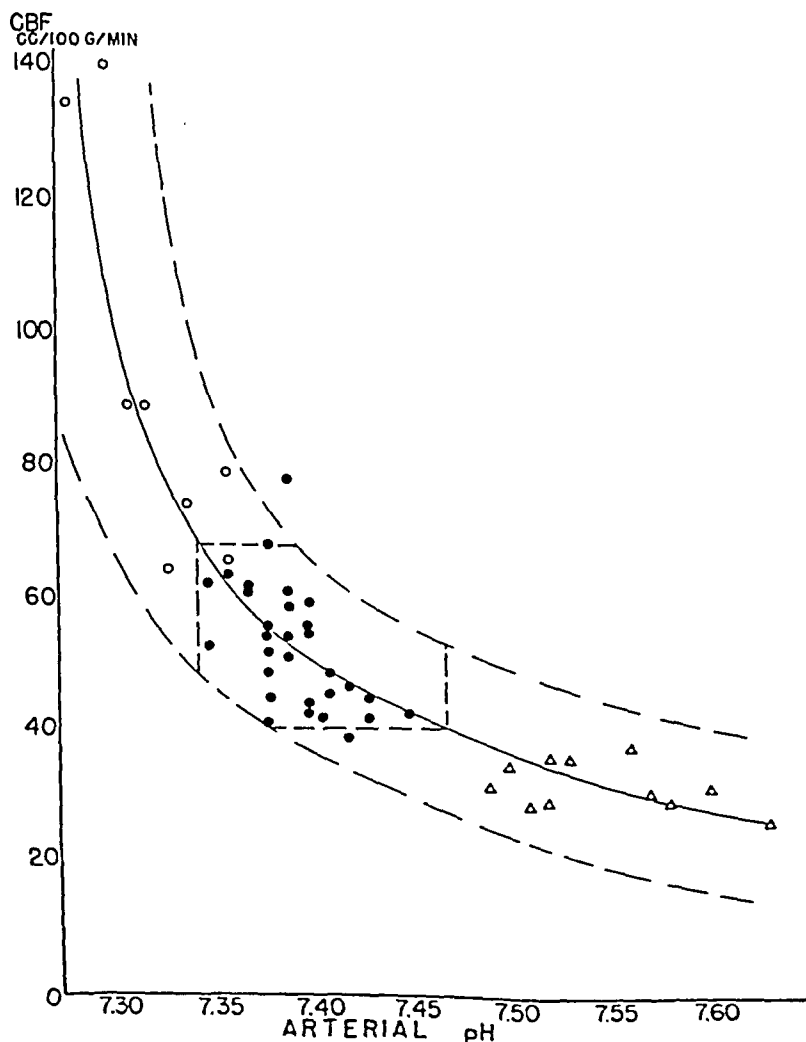


FIG. 2. THE RELATIONSHIP BETWEEN CEREBRAL BLOOD FLOW AND ARTERIAL pH. The latter was varied from the normal (dots) by hyperventilation (triangles) or by inhalation of 5-7% CO_2 (open circles). The broken curves bound 98% of the observations while the central polygon encloses 94% of the normal values.

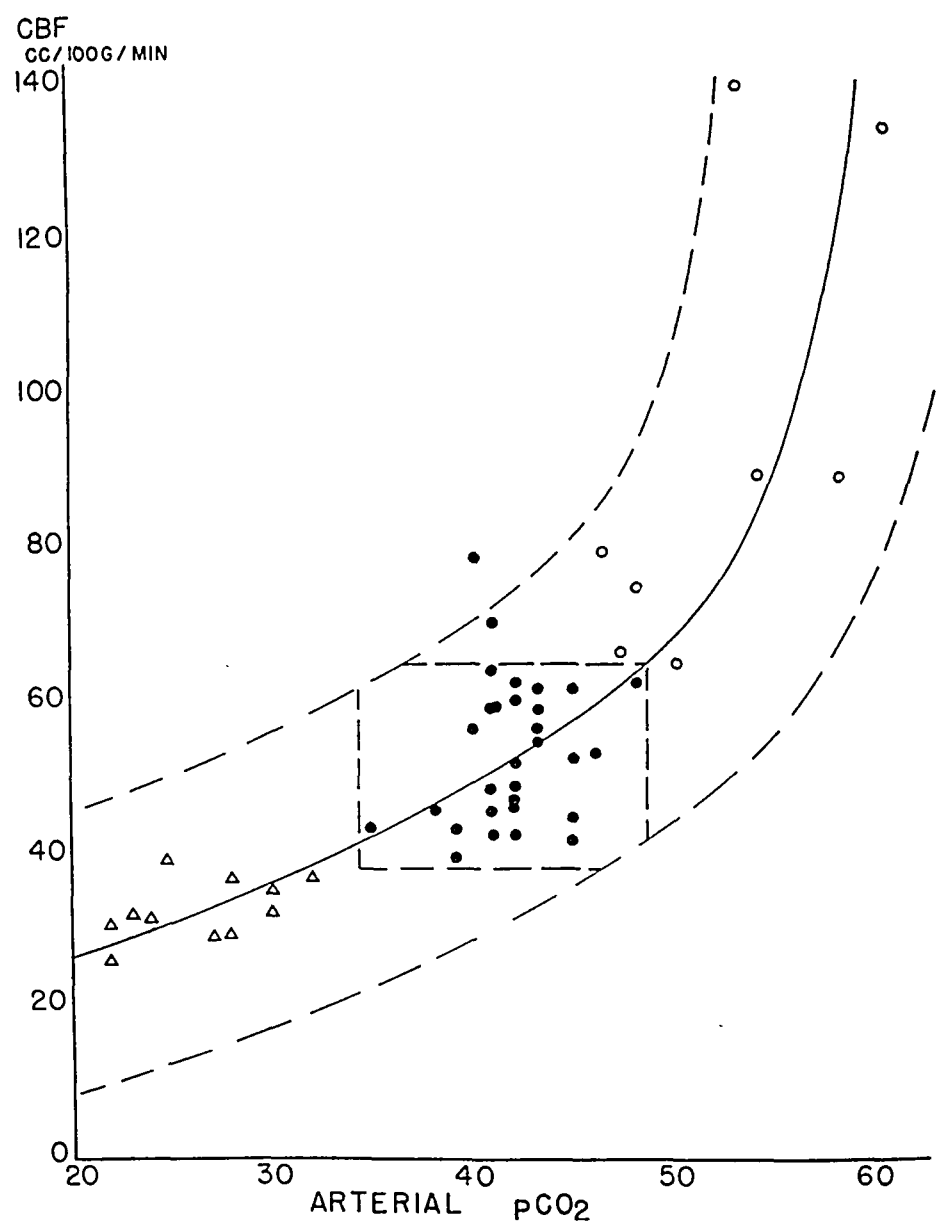


FIG. 3. THE RELATIONSHIP BETWEEN CEREBRAL BLOOD FLOW AND ARTERIAL CO₂ TENSION

Symbols and construction are similar to those in Figure 2.

verse, that a change in cerebral oxygen utilization does not effect consciousness, for when the former is significantly diminished, mental function deteriorates (15 to 17).

These data offer evidence in man for a delicate control over the internal environment of the brain achieved through the intrinsic regulation of the tone of cerebral vessels. Such a homeostatic mechanism has previously been suggested (11, 18 to 20). An examination of the blood changes which occurred in these studies (Table I) shows that in each case where an abnormal change has been imposed on the arterial blood the change is considerably damped in the internal jugular blood

which represents a closer approximation to the state of affairs in the brain itself. Thus in hyperventilation where arterial CO₂ content and tension and pH were changed by 8.4 vol. %, 19 mm., and 0.16 units, respectively, the corresponding changes in internal jugular blood were 3.4 vol. %, 14 mm., and 0.13 units. The inhalation of 5-7% CO₂ produced changes in arterial CO₂ content and tension and pH of 3.0 vol. %, 9 mm., and 0.05 units while internal jugular blood showed corresponding changes of only 0.6 vol. %, 4 mm., and 0.02 units. High oxygen inhalation produced a 1.4 vol. % increase in arterial oxygen content as contrasted with only a 0.5 vol. % increase in

cerebral venous blood. Anoxia produced a reduction in arterial oxygen content of 6.3 vol. %, but only a 4.1 vol. % decrease in the venous value. Aside from establishing the intrinsic nature of these responses our data throw no further light on the mechanism which mediates them, whether it be by direct action on the vessel walls or by an intrinsic reflex via the well-established cerebral vasodilator nerves (21). In the cases of CO₂ inhalation and hyperventilation it is impossible to decide whether the prime stimulus is CO₂ tension or the concomitant change in hydrogen ion concentration. Figures 2 and 3 show that very good correlations exist between cerebral blood flow and either arterial pCO₂ or arterial pH. There is evidence in our data on patients in diabetic acidosis (16) where an increase in hydrogen ion concentration is associated with a decrease in pCO₂ that beyond certain limits arterial pH may become the dominant factor in cerebrovascular tone as well as respiration.

In the application of this concept to the adjustment of cerebral blood flow to local metabolic needs, which previous work has shown to exist (20) it is a fortunate fact that increased pCO₂ and hydrogen ion concentration as well as a decrease in pO₂, all products of metabolism, appear individually capable of producing vasodilatation and therefore of maintaining the adjustment of flow to metabolism in the brain, although it is probable that this adjustment is achieved by a summation of these and many other vasodilator products of metabolism.

SUMMARY

1. The effects of the inhalation of 5-7% CO₂, 85-100% O₂, and 10% O₂ were studied on the composition of arterial and internal jugular blood; on blood flow, oxygen consumption, and vascular resistance of the brain; on cardiac output and blood pressure.

2. CO₂ inhaled in concentrations of 5-7% produces an increase in cerebral blood flow averaging 75%. O₂ inhaled in concentrations of 85-100% is associated with a reduction in cerebral blood flow of 13%, while 10% O₂ produced an increase of 35% in this function. These changes are statistically significant.

3. Calculation of cerebrovascular resistance indicates that in every case the change in blood flow

is due to a change in the vascular resistance of the brain.

4. Cerebral oxygen consumption is not significantly altered by changes in the composition of inspired air over the ranges studied.

5. Mean arterial blood pressure rose significantly during the CO₂ and high O₂ inhalations and fell slightly with 10% O₂.

6. The only significant change in cardiac minute volume was an increase which occurred during 10% O₂ inhalation and resulted from an increase in rate rather than stroke volume.

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THE EFFECTS OF INCREASED INTRACRANIAL PRESSURE ON CEREBRAL CIRCULATORY FUNCTIONS IN MAN¹

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The effect of increased intracranial pressure on cerebral blood flow has been the object of very few clinical studies. In accordance with the Monroe-Kellie-Cushing Doctrine, it would be presumed that increased intracranial pressure would increase cerebrovascular resistance and thereby decrease cerebral blood flow. However, Williams and Lennox (1) in 1939 concluded on the basis of cerebral arteriovenous oxygen differences that cerebral blood flow was practically unaffected by a rise in cerebrospinal fluid pressure. Courtice (2) also working with humans and using a similar technique came to a different conclusion: that there was a slowing of blood flow through the brain in certain types of brain tumor associated with increased intracranial pressure. More recently Ferris (3), using a plethysmographic meas-

urement of relative intracranial blood flow, reported a diminution in cerebral blood flow in two subjects when intracranial pressure was artificially raised above 350 mm. of water.

The development of a technique which appears capable of yielding quantitative information on cerebral blood flow in man (4, 5) makes available more definitive information on the effects of increased intracranial pressure on this important function.

METHODS

The patients studied were 13 in number; all but one were suffering from brain tumors. Cerebrospinal fluid pressures were determined in millimeters of water above the horizontal cerebrospinal axis through a needle inserted into the lateral ventricle or lumbar subarachnoid space. Mean arterial pressure was measured from the femoral artery by a damped mercury manometer attached directly to the arterial needle. Cerebral blood flow (CBF) was determined by the nitrous oxide technique previously described (5), using 21% O₂, 64% N₂, and 15% N₂O as the inhalation mixture. From this value and the cerebral arteriovenous oxygen difference or the mean arterial blood pressure, cerebral oxygen utilization

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TABLE I

Patient	Age	Sex	Mental state*	Arterial				Int. jugular				Mean art. B. P.	CSF pressure
				CO ₂ content	CO ₂ tension	O ₂ content	pH	CO ₂ content	CO ₂ tension	O ₂ content	pH		
				vol. %	mm. Hg	vol. %		vol. %	mm. Hg	vol. %		mm. Hg	mm. H ₂ O
Gre.	50	M	C	50.0	38	18.7	7.49	56.9	45	11.6	7.43	95	430
Fri.	40	M	C	52.0	37	11.0	7.45	56.5	41	6.7	7.43	85	100
Ger.	38	M	C	48.9	37	15.6	7.44	54.3	46	9.2	7.39	97	415
Her.	40	M	C	38.2	34	18.3	7.38	43.8	45	12.3	7.31	160†	295
Smi.	43	F	C	45.7	36	14.0	7.42	52.4	45	7.3	7.37	111	170
Ant.	38	F	C	45.1		16.6		49.5		12.5		85	130
Woo.	52	F	U	44.8	38	16.6	7.40	51.2	45	9.4	7.36	130	840
Woo.	52	F	U	50.1	39	17.3	7.43	57.0	50	9.8	7.38	125	820
Fos.	52	M	U	44.9	32	17.8	7.50	53.2	42	8.0	7.42	122	620
Bur.	49	M	U	43.7	34	16.6	7.43	49.3	39	12.2	7.42	97	350
Haw.	48	M	C	48.9	40	18.1	7.42	55.2		10.9		95	460
Pet.	33	M	U	45.6	41	22.5	7.39	55.0	57	13.4	7.32	117	545
Bal.	48	M	U	46.0	29	13.0	7.53	50.7	36	7.2	7.50	95	325
Roo.	41	M	C	48.5	40	13.6	7.40	52.6	46	8.8	7.36	105	410
Mean Normal Values				49	41	18	7.40	55	51	12	7.34	86	150

* C = Conscious. U = Unconscious.

TABLE I—Continued

Patient	Cerebral					Resp. min. vol.	Diagnosis	Localization
	CBF	CMR O ₂	CVR	A-V O ₂	R.Q.			
	cc./100 g./min.	cc./100 g./min.	$\frac{\text{mm. Hg}}{\text{cc./100}} \frac{\text{g.}}{\text{min.}}$	vol. %		l/min.		
Gre.	55	3.9	1.7	7.1	0.97	8.9	Metastatic carcinoma	Supratentorial
Fri.	61	2.8	1.4	4.4	1.03	9.1	Cystic oligodendroglioma	Left frontal
Ger.	53	3.4	1.8	6.4	0.84	12.0	Glioma	Left temporo-parietal
Her.	47	2.9	3.4†	6.1	0.93	8.6	Astrocytoma	Right parieto-occipital
Smi.	47	3.1	2.4	6.7	1.00	17.0	Glioma	Left fronto-parietal
Ant.	52	2.2	1.6	4.2	1.05	4.5	Suspected brain tumor— (multiple sclerosis)	.
Woo.	31	2.2	4.2	7.2	0.90		Tuberculoma	Posterior fossa
Woo.	33	2.5	3.8	7.6	0.92		Tuberculoma	Posterior fossa
Fos.	33	3.2	3.7	9.8	0.85		Glioblastoma multiforme	Left fronto-parietal
Bur.	39	1.7	2.5	4.4	1.27	9.6	Metastatic carcinoma	Posterior fossa
Haw.	40	2.9	2.4	7.2	0.87		Glioblastoma multiforme	Right temporo-parietal
Pet.	33	3.0	3.5	9.2	1.03	7.1	Astrocytoma	Cerebellar
Bal.	45	2.6	2.1	5.8	0.81		Glioma	Left fronto-parietal
Roo.	64	3.1	1.6	4.8	0.86		Meningioma	Left parieto-occipital
Mean Normal Values	54	3.3	1.6	6.3	0.99	8.1		

† This patient was known to have essential hypertension over a period of many years. These figures have been excluded from the correlations.

(CMR_{O₂}) and cerebrovascular resistance (CVR) were calculated (5). Respiratory minute volume was measured by means of a tightly fitting mask and a Tissot spirometer.

RESULTS

The pertinent data obtained in these studies are presented in Table I. Some of the relationships

among the data are indicated by the scatter diagrams (Figures 1–4). There are good correlations between cerebrospinal fluid pressure and mean arterial blood pressure, cerebrovascular resistance, and cerebral blood flow, as well as a satisfactory correlation between cerebral blood flow and mean arterial blood pressure. Some of these correlations could have been deduced from

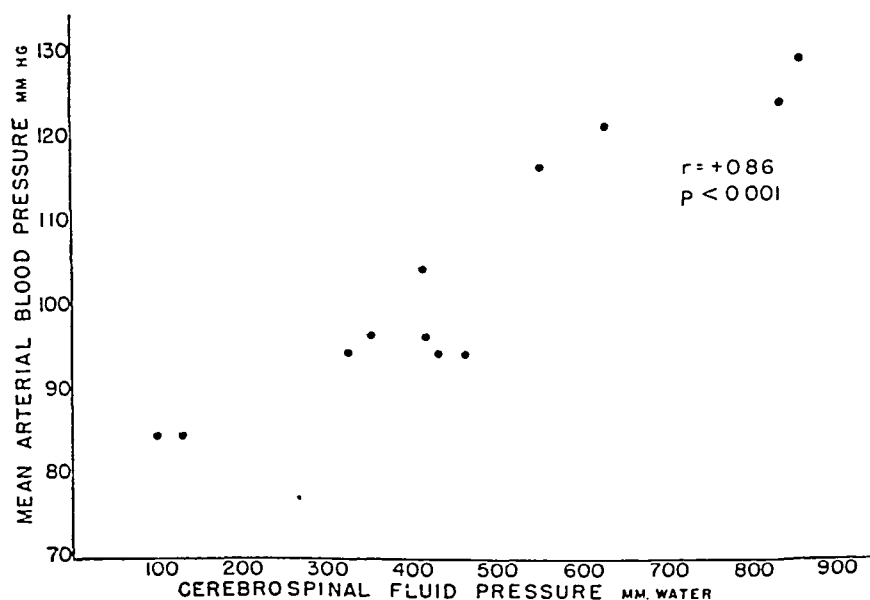


FIG. 1. THE RELATIONSHIP BETWEEN INTRACRANIAL PRESSURE AND MEAN ARTERIAL BLOOD PRESSURE

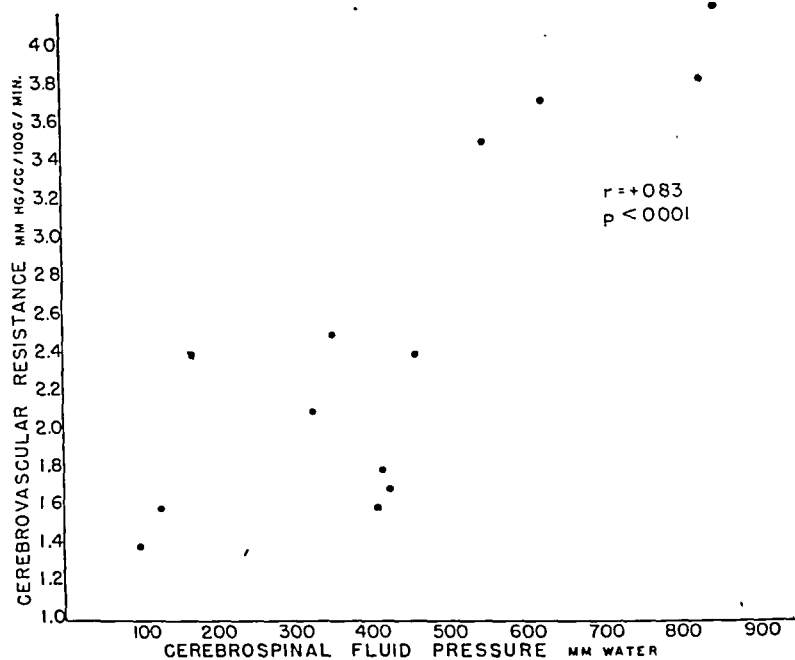


FIG. 2. THE RELATIONSHIP BETWEEN INTRACRANIAL PRESSURE AND CEREBROVASCULAR RESISTANCE

the others; their inclusion is simply for the sake of clarity. In corroboration of Ferris' findings (3), there appears to be a critical level which intracranial pressure must attain before significant

cerebral circulatory embarrassment occurs. In our studies this level is close to 450 mm. of water (33 mm. Hg); pressures below that value in nine patients were associated with an insignificant re-

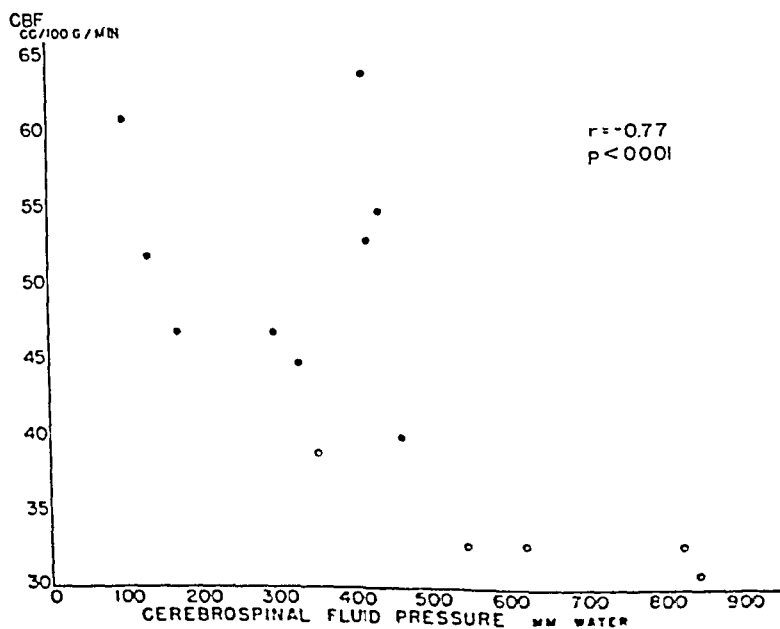


FIG. 3. THE RELATIONSHIP BETWEEN INTRACRANIAL PRESSURE AND CEREBRAL BLOOD FLOW

Open circles represent comatose patients.

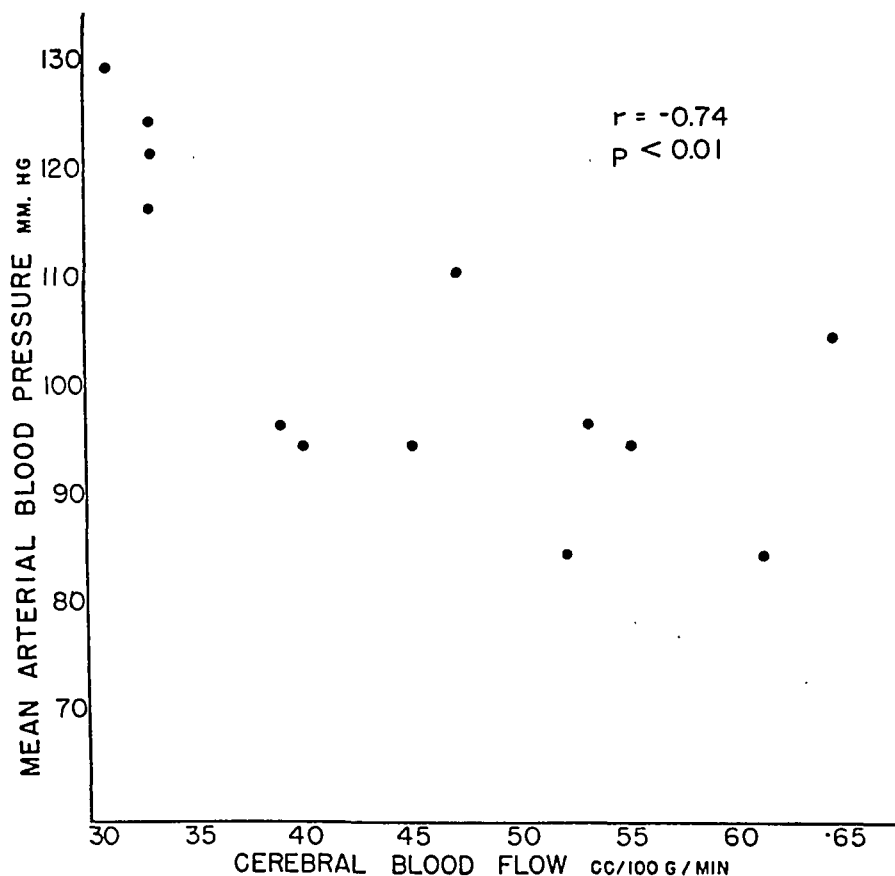


FIG. 4. THE RELATIONSHIP BETWEEN CEREBRAL BLOOD FLOW AND MEAN ARTERIAL BLOOD PRESSURE

duction in cerebral blood flow (51 as compared with a normal value of 54 cc./100 g./min.) while there was a marked diminution in this function in the five cases where cerebrospinal fluid pressures were above 450 mm. (34 cc./100 g./min.). This difference between the two groups is statistically highly significant ($p < 0.001$). There was a distinctly lower average cerebral blood flow in the four observations on patients with posterior fossa tumors (35 cc./100 g./min.) than for the nine with cerebral hemispheric lesions (49 cc./100 g./min.). While this could be due to interference with venous drainage, as Courtice suggests (2), it appears more likely to be related to the greater pressure associated with posterior fossa lesions, since these showed no variation from the general correlation of cerebral blood flow versus cerebrospinal fluid pressure. Although those patients in whom such measurements were made exhibited an increase in respiratory minute volume, and the patients as a whole showed some depression of arterial $p\text{CO}_2$ indicative of respira-

tory stimulation, these showed no correlation with intracranial pressure.

The patients were classified into two groups, conscious and comatose, on the basis of obvious objective indications such as response to questions and other stimuli. There was a good correlation between mental state and cerebral oxygen consumption as is found in other clinical conditions (6, 7). The conscious group yielded a mean value for CMR_{O_2} of 3.1 cc./100 g./min. while the comatose group averaged 2.5 cc./100 g./min., a difference which is statistically significant ($p = 0.05$). The mean value for this function in normal young men is 3.3 cc./100 g./min. (5).

The effects produced on cerebral blood flow, metabolism and vascular resistance by acutely reducing the intracranial pressure is the subject of another report (8).

DISCUSSION

At the turn of the present century Harvey Cushing reported some observations on a phenomenon which has since been closely associated with his

name (9, 10). By several precise experiments he demonstrated that an increase in intracranial pressure, acutely induced, was associated with a rise in blood pressure to a level somewhat above that in the cerebrospinal system. He showed further that this hypertension was the result of a peripheral vasoconstriction, mediated through tracts in the cervical cord arising in a medullary vasomotor center. He postulated that stimulation of the vasomotor center was the result of medullary ischemia rather than the activation of a sensory reflex. Not much has been added to our knowledge of this phenomenon in the intervening 47 years.

The present study reveals in man those phenomena which Cushing induced in animals and corroborates in many respects the hypothesis by which he explained them. Figure 1 shows the excellent correlation ($r = 0.86$) which is found between intracranial pressure and mean arterial blood pressure, an observation which is now commonplace. There is one difference, however, between this relationship in patients chronically exposed to an increased cerebrospinal fluid pressure and the acute Cushing phenomenon, in that the patients exhibit a progressive increase in blood pressure as intracranial pressure rises, whereas in the experimental counterpart blood pressure does not begin to rise until the cerebrospinal fluid pressure closely approaches it. The acuteness of the animal experiments and the depressant effect of anesthesia on the mechanisms involved may possibly explain this difference.

It is of interest to seek, in these studies on man, evidence for the mechanism whereby blood pressure rises *pari passu* with an increase in intracranial pressure. Quantitative measurement of cerebral blood flow permits an evaluation of cerebrovascular resistance (CVR) in terms of the pressure head necessary to achieve a unit of cerebral blood flow. In the correlation shown in Figure 2 it is seen that this function increases in fairly exact proportion to the increase in cerebrospinal fluid pressure ($r = 0.83$). This is entirely in accord with expectation; the capillaries of the brain, being freely collapsible, transmit any external pressure in opposition to the pressure head of the flowing blood. An exact analogy to this phenomenon is seen in the familiar variable re-

sistance employed in the heart-lung preparation. It is very likely that this increase in cerebrovascular resistance represents the primary effect of high intracranial pressure. Were no compensatory mechanisms operating, this should result in a progressive restriction of cerebral blood flow. That this does occur is evident from Figure 3 showing a fairly good correlation ($r = -0.77$) between intracranial pressure and cerebral blood flow. The relationship, however, is more complex than appears to be the case in this single correlation. More careful scrutiny reveals the fact that up to a cerebrospinal pressure of 450 mm. of water this correlation is poor, indicating possibly, that in this region the compensatory rise in arterial pressure is sufficient to overcome the increased resistance whereas this adjustment falls short in the regions of higher pressure. These findings agree with those of Ferris (3) who found an intracranial pressure of similar magnitude necessary before relative intracranial blood flow was measurably restricted. They also demonstrate in man the implication of experiments on animals in which, by a study of circulation times (11) or pial vessel diameters (12), it was concluded that increased intracranial pressure caused a restriction in cerebral blood flow. The patients who failed to compensate and exhibited a reduction of cerebral blood flow below 40 cc./100 g./min. were all comatose. Whether consciousness failed because of the severe restriction in blood flow, or whether the compensation failed because of the generalized neuronal depression manifested in unconsciousness cannot be decided from these data. Our demonstration of a reduced cerebral blood flow in these comatose patients, although reasonable, is quite at variance with Williams and Lennox (1) who concluded that there was an increased cerebral blood flow in four deeply comatose patients in their series on raised intracranial pressure. They based this conclusion on their finding of a reduced cerebral arteriovenous oxygen difference in these individuals, neglecting the more likely alternative that deeply comatose patients might be expected to have a decreased cerebral metabolism.

Williams and Lennox found in their complete series of seven patients a mean arteriovenous oxygen difference of 6.6 vol. %; Courtice's 24 patients (2) yield a mean value of 6.7 vol. %, as do our pa-

tients. All of these averages are above the normal mean of 6.3 vol. %, which simply means that in this condition there is a slightly decreased supply of oxygen with respect to its utilization. Since the latter is not likely to be increased in this condition this suggests, but by no means demonstrates, a decrease in cerebral blood flow. The arteriovenous oxygen difference, being a function of both blood flow and oxygen consumption is in itself a measure of neither.

Cushing further postulated that this compensatory rise in arterial pressure is the result of medullary ischemia; our findings are compatible with this idea. Figure 4, showing a fairly good correlation between mean arterial blood pressure and cerebral blood flow ($r = -0.74$), suggests that cerebral ischemia plays some role in the hypertension. Certainly, the possibility that the high blood pressure could cause the reduced blood flow is hardly tenable. The manner in which medullary ischemia might effect a rise in blood pressure is quite possibly by way of a relative asphyxia of the vasomotor center. There is at hand no means of studying directly the internal environment of the human medulla. The closest approach possible at present is an examination of the composition of cerebral venous blood which may be expected to reflect but by no means to define general changes in the *milieu interne* of cerebral cells. Where the changes were brought about by alteration in blood flow rather than by a changed internal metabolism this parallelism might be especially valid. An examination of the relationship of the arterial blood pressure to cerebral venous $p\text{CO}_2$, hydrogen ion concentration, and oxygen saturation shows a fair correlation in each case ($r = +0.50$, $+0.58$ and -0.58 , respectively) in such a direction as to imply a rise in arterial blood pressure concomitant with progressive central asphyxia.

It thus appears that increasing intracranial pressure in these patients is associated with an increase in cerebrovascular resistance, a tendency towards restricted cerebral blood flow and progressive cerebral asphyxia. There occurs *pari passu* a rise in arterial blood pressure correlated with the increasing intracranial tension, the restricted circulation and the available indications of central asphyxia.

These findings in man are entirely in accord

with Cushing's hypothesis of medullary ischemia and complement hitherto available knowledge with quantitative data on cerebral blood flow, metabolism and cerebrovascular resistance.

SUMMARY

1. The relationships between intracranial pressure and cerebral blood flow, oxygen consumption and cerebrovascular resistance were studied in 13 patients.
2. The rise in cerebrospinal fluid pressure produced by brain tumor is associated with a progressive increase in cerebrovascular resistance, in mean arterial blood pressure, and above a certain level, with a definite decrease in cerebral blood flow.
3. These findings substantiate in man the hypothesis of medullary ischemia elaborated by Cushing on the basis of animal experiments.

ACKNOWLEDGMENT

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THE BLOOD FLOW AND OXYGEN CONSUMPTION OF THE HUMAN BRAIN IN DIABETIC ACIDOSIS AND COMA¹

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Despite recent gratifying advances in our knowledge of carbohydrate metabolism, diabetic coma remains an important medical emergency and our understanding of its fundamental pathogenesis and rational treatment is far from complete. The sequence of events leading from disturbed carbohydrate utilization through acidosis, coma and death has been variously defined and important roles have been assigned at one time or another to ketosis, acidosis, dehydration, circulatory failure, and cerebral anoxia.

In an effort better to define these fundamental derangements and their inter-relationships, the brain was chosen as an important object of study since its integrity is so intimately associated with survival. Clinicians who have studied diabetic acidosis are agreed that cerebral function as reflected in mental state is closely correlated with the severity of the disease and its prognosis (1-6). The central nervous system, apparently dependent on carbohydrate for its normal source of energy (7 to 9), would be expected significantly to reflect disturbances in the metabolism of this foodstuff. For many reasons recently recapitulated by Soskin and Levine (10), the results of extensive *in vitro* studies may not rigorously be applied to the metabolism of tissues in the living organism so that inferences drawn from work with the Warburg apparatus require validation by studies on living organs *in situ*. Such studies are comparatively rare because suitable methods have been developed only in the past few years.

The recently devised nitrous oxide method for the quantitative measurement of cerebral blood flow in man (11, 12) makes possible calculation of the utilization or production by the brain of any

substance susceptible of accurate analysis in arterial and cerebral venous blood. In applying this method to diabetic acidosis it was decided first to obtain an overall estimate of cerebral metabolism by determination of the oxygen consumption of the brain and, if possible, an indication of the factors which may influence it in this condition. If this approach appeared feasible, it was hoped that analytical techniques could be refined or developed to permit examination of more specific phases of cerebral metabolism and the aberrations associated with diabetic acidosis. The present report concerns itself largely, therefore, with cerebral oxygen utilization.

METHODS

Members of the investigating team were notified as soon as a diagnosis of diabetic coma or severe acidosis was made in the receiving ward and confirmed by blood sugar and CO₂ determinations. Studies were not attempted where too great a delay in treatment would have resulted, where the acidosis was not very severe, or if the patient was moribund. There was no difference between the mortalities in this series and in comparable patients on the same services before this investigation was undertaken.

Respiratory minute volume was measured in a Tissot spirometer attached to a well-fitted face mask equipped with expiratory and inspiratory valves. Mean arterial blood pressure was read from a damped mercury manometer communicating with a needle in the femoral artery. The nitrous oxide method (12) employing a gas mixture of 15% N₂O, 21% O₂, 64% N₂, was used for measuring cerebral blood flow.

Blood samples were analyzed for oxygen and carbon dioxide (13), hemoglobin (14), glucose (15), total ketones (16), protein (17), chlorides (18), total base (19) and urea nitrogen (20). Hydrogen ion concentration was measured potentiometrically at 37° under anaerobic conditions using a glass electrode. Values for CO₂ tension were calculated from CO₂ content and pH by means of nomograms (13).

Immediately after the required blood samples were taken, treatment was begun. In seven instances the studies above were repeated at some time (varying from two to 48 hours) after the initiation of treatment.

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RESULTS

Table I contains the pertinent clinical data on the 14 patients on whom practically complete studies were performed, while the biochemical and physiological data are presented in Tables II and III.

Patients. These were fairly evenly distributed with respect to sex and varied in age from 22 to 67. The gaps in the anamnesis result from the fact that many of these patients were unknown or neglected diabetics brought to the hospital in coma with no history obtainable.

Mortality. The mortality for the series of 14 patients was 43%. This figure which appears quite high is completely in line with mortality figures reported for similar patients in this hospital (1) and elsewhere (3 to 5). It agrees well with a

predicted mortality of 38% calculated for this series according to the formula devised by Collen (5). After a little experience with such cases it becomes obvious that there are really two fairly distinct clinical entities involved. One group, which we have classified as severe diabetic acidosis, is tolerating drastic biochemical derangements in homeostasis which have not yet seriously involved the brain and other vital organs. These patients are conscious, though often confused, and suffer little circulatory embarrassment. Their response to insulin and parenteral fluids is gratifying and the mortality is comparatively low (13% in our series, 24% in this hospital for the four years preceding this investigation, 14-28% in the reports of others who dealt with similar patients

TABLE I
Clinical data

Patient	Age	Sex	Usual daily dose of insulin	Duration of drowsiness	Duration of vomiting	Complications	Outcome
	yrs.		units	hrs.	hrs.		
Severe diabetic acidosis admission data							
J. S.	24	M	70		6		Recovered
M. M.	46	F	100		24		Recovered
M. G.	64	F					Died
T. H.	67	M	0				Recovered
C. B.	30	M	30	72	48	Pyelitis, hydronephrosis	Recovered
P. S.	40	F	23		48	Grippe	Recovered
C. C.	24	M	50				Recovered
A. B.	52	F	55				Recovered
Mean	43						

Diabetic coma admission data

M. B.	41	M		(coma) 24		Hepatic cirrhosis	Died
P. B.	60?	M		96		Acute pancreatitis	Died
F. A.	32	M		96		Erysipelas	Died
A. D.	38	F					Died
F. R.	54	F	30		30		Died
M. O.	22	F	90	48		Dental abscess	Recovered
Mean	41						

Treatment received between cerebral blood flow studies

Patient	Elapsed time between studies	Plasma liters	Physiologic saline liters		Sodium bicarbonate gm.		Insulin units	Glucose gm.	Water liters
	hrs.	I.V.	I.V.	S.C.	P.O.	I.V.		I.V.	I.V.
J. S.	2		0.7		14		150		1
M. M.	5		1.4	2	10	10	450		
T. H.	15		1	2	16		500	50	1
C. B.	48	0.5		2	12		400	50	1
C. C.	3					11	200		0.5
P. B.	2	1.5		1		19	300	50	1
A. D.	2	1.5	2			15	500		

TABLE II
Blood constituents

Patient	Arterial										Internal jugular																
	Glucose	Total ketones	Protein	BHCO ₃	Cl	Total base	Hemo- globin	O ₂ saturation	O ₂ content	CO ₂ content	pCO ₂	pH	Urea nitrogen	O ₂ content	CO ₂ content	pCO ₂	pH										
																		mg./100 cc.	gm./100 cc.	m.Eq./l.	m.Eq./l.	gm./100 cc.	%	vol. %	mm. Hg	vol. %	mm. Hg
Severe diabetic acidosis admission data																											
J. S.	217	38.5	7.3	16.5	86	148	16.7	90	20.2	32.3	30	7.36	11	10.3	40.6	40	7.32										
M. M.	783	88.8	9.5	4.8	88	142	12.7	90	15.3	10.2	15	7.12		11.1	14.3	21	7.10										
M. G.	698	75.6	7.7	6.2	103	127	16.5	86	19.0	13.2	24	7.03	39	14.3	17.3	32	7.01										
T. H.	668	90.6	6.5	6.1	94	129	12.2	92	14.9	13.1	17	7.17	68	9.3	18.6	25	7.14										
C. B.	744	83.5	7.4	3.3	82	136	15.2	99	20.1	7.4	17	6.91	30	13.2	13.2	31	6.90										
P. S.	620	44.6	9.0	3.1	107	137	15.0	98	19.7	6.6	11	7.06	22	14.1	11.9	21	7.03										
C. C.	231	42.7	11.9	5.5	92	137	16.3	95	20.6	11.0	14	7.21	16	14.3	17.2	22	7.19										
A. B.	692			5.4	91		12.7	96	16.3	11.2	14	7.20	61	9.4	17.3	25	7.12										
Mean	582	66.3	8.5	6.4	93	137	14.7	93	18.3	13.1	18	7.13	32	12.0	18.8	27	7.10										
Diabetic coma admission data																											
M. B.	544	132.5	8.0	3.7	113		17.5	91	21.2	7.4	15	7.00	23	20.1	8.2	17	6.98										
P. B.	482	85.5	6.6	5.0	102	154	12.7	105	17.9	10.2	15	7.14	52	13.6	14.4	21	7.11										
F. A.	390	99.0	6.8	7.5	81	142	13.8	90	16.6	15.3	23	7.12		10.9	20.0	31	7.09										
A. D.	592	71.4	6.9	5.1	94	151	12.4	91	15.1	11.2	23	6.96	22	12.7	13.5	28	6.94										
F. R.	669	154.0	6.7	3.8	102	149	14.8	93	18.3	8.6	22	6.86	23	16.6	10.2	27	6.83										
M. O.	630	47.7	8.4	2.9	105	148	15.4	91	18.7	6.0	18	6.80	20	16.0	8.2	22	6.80										
Mean	552	98.4	7.3	4.7	100	149	14.4	94	18.0	9.8	19	6.98	28	15.0	12.4	24	6.96										
Post treatment data																											
J. S.	158	25.1	5.4	31.7	94	151	13.8	90	16.7	60.6	42	7.50		11.9	66.6	47	7.48										
M. M.	278	75.6	7.6	10.8	96	156	12.0	92	14.7	21.7	19	7.37		8.9	27.1	27	7.32										
T. H.	145	25.2	7.3	28.2	104	155	10.4	94	13.1	58.6	45	7.42	18	7.9	63.1	52	7.39										
C. B.	173	5.0	5.8	28.5	99	152	13.2	92	16.2	57.6	43	7.44		8.1	64.9	56	7.38										
C. C.	166	42.5	9.5	11.5	96	134	15.4	94	19.3	22.3	21	7.35	14	12.1	29.6	29	7.32										
P. B.	468	73.2	6.3	13.8	110	199	9.4	103	13.0	29.2	23	7.40		9.5	32.4	24	7.43										
A. D.	421	60.6	6.7	8.4	94		10.1	91	12.3	18.4	20	7.24		10.4	20.1	24	7.20										
Approximate normal values	90	1.5	7.2	25.0	100	155	14.0	96	18.0	49.0	40	7.40	15	11.0	55.0	51	7.34										

TABLE III
Cerebral and other physiological data

Patient	Mental state	Cerebral					Respiratory			Mean arterial B. P.	Pulse rate per min.	Rectal temp. °F.
		A-V O ₂	CBF	CMR O ₂	CVR	RQ	Minute volume	Tidal volume	Rate per min.			
		vol. %	cc./100 g./min.	cc./100 g./min.	$\frac{\text{mm. Hg}}{\text{cc./100 g./min.}}$		liters	cc.				
Severe diabetic acidosis admission data												
J. S.	Confused	9.9	41	4.1	1.8	0.84	7.5	500	15	74	130	95
M. M.	Confused	4.2	58	2.4	1.4	0.98	33	920	36	79	128	100
M. G.	Confused	4.7	55	2.6	1.1	0.87	35	880	40	58	120	102
T. H.	Confused	5.6	32	1.8	3.8	0.98	21	1100	19	120	110	99
C. B.	Confused	6.9	36	2.5	2.4	0.84	26	1300	20	86	116	100
P. S.	Confused	5.6	50	2.8	2.1	0.96	31	1200	25	107	94	99
C. C.	Confused	6.4	40	2.6	2.1	0.99	10.7	400	26	84	110	99
A. B.	Confused	6.9	44	3.0	1.8	0.88	16.9	770	22	80	110	98
Mean		6.3	45	2.7	2.1	0.92	23	880	25	86	115	99
Diabetic coma admission data												
M. B.	Uncon.	1.2	63	0.8	1.3	0.70				80	160	96
P. B.	Uncon.	4.3	35	1.5	1.8	0.98	28.4			64	120	96
F. A.	Uncon.	5.7	35	2.0	1.0	0.83	23.9			34	105	101
A. D.	Uncon.	2.4	80	1.9	0.8	0.96				65	110	98
F. R.	Uncon.	1.8	101	1.8	0.8	0.91	20	850	24	82	106	95
M. O.	Uncon.	2.8	78	2.1	0.9	0.81	25	780	32	72	135	97
Mean		3.0	65	1.7	1.1	0.87				66	123	97
Post treatment data												
J.S.	Alert	4.9	63	3.1	1.4	1.25	7.7	550	14	90		
M. M.	Alert	5.8	54	3.1	1.1	0.93	18.7	750	25	62	112	
T. H.	Alert	5.2	42	2.2	2.0	0.87	10	560	18	86		
C. B.	Alert	8.1	40	3.2	2.3	0.90	5	560	9	92	88	
C. C.	Confused	7.2	45	3.2	1.9	1.02	6.8	340	20	84	100	
P. B.	Uncon.	3.5	48	1.7	1.6	0.92	26.6			76		
A. D.	Confused	2.0	120	2.4	0.7	0.90				83		
Normal values		6.3	54	3.3	1.6	0.99	8	500	16	86		

[1 to 5]). The other type is characterized by unconsciousness, frequently associated with circulatory depression. These alone we have felt justified in classifying as diabetic coma. Satisfactory correction of the chemical disturbances in the blood of these patients has little effect on the course of the disease and the mortality is almost always high (83% in our series, 80% in the four years preceding this investigation, 31%, 58%, 70%, 73%, 75% and 81% in other series [1 to 6]). The overall mortality of any series of cases of diabetic acidosis and coma depends far more on the relative frequency of these two groups in the series than on the minor differences which

may occur in a treatment so universally accepted and used. It should be pointed out that a diagnosis of coma on the basis of the plasma CO₂ combining power, rather than on mental state, does not differentiate between these two groups and may be misleading from the point of view of prognosis and therapeutic results. A case in point is the overall mortality of 11% reported by one group (6) in a series of 525 cases of "diabetic coma" based on the plasma CO₂, only 18% of whom were unconscious. The mortality in these 93 unconscious patients, however, was only 31%, an enviable record in itself. It is worthy of note that all the deaths in our series were in patients

who had never attended the Metabolic Clinic of the hospital, and in five of the six deaths the presence of diabetes had been unknown.

Acid-base balance. There was a marked reduction in the arterial carbon dioxide content, tension and pH, a picture of uncompensated metabolic acidosis. The lowest value for pH (6.80) was obtained in a patient who subsequently recovered. There is surprisingly little difference between the "acidosis" and "coma" patients on the basis of these three functions. In all cases studied during or after treatment, which included moderate amounts of sodium bicarbonate, there was a striking recovery of pH to normal or nearly normal levels even though the plasma bicarbonate lagged far behind. The reason for this is seen in the slow return of the CO_2 tension to normal indicative of only a gradual reduction of hyperventilation in response to the improved pH. The practical implications of this with respect to the question of alkali administration are important. There is some controversy at present on the advisability, amount and route of administration of bicarbonate in the treatment of diabetic acidosis. Unfortunately the criterion of acidosis and response to treatment has been the plasma CO_2 combining power, at best a very rough guide. Actually it is the ratio of bicarbonate to CO_2 tension (pCO_2) which is important and this can best be determined by measurement of the pH which represents the resultant of all the blood buffer systems. Since the pCO_2 does not rise *pari passu* with the administered bicarbonate the pH will be brought to normal much sooner than will the blood bicarbonate concentration. Thus, any attempt to administer alkali rapidly in quantities calculated to restore the CO_2 combining power to a normal level can only result in a severe alkaline shift of the pH until the relatively sluggish respiratory adjustments are made. Our results demonstrate that it is possible to bring the arterial pH within normal limits quickly by means of the intravenous administration of amounts of bicarbonate small enough to raise the blood CO_2 content only 10 or 15 vol. %. For example, patients ? B., M. M. and C. C. showed a normal arterial pH with an alkali reserve less than half the normal after two to five hours of treatment. In the case of A. D. the arterial pH rose from 6.96 to 7.24 with a change in BHCO_3 of from 5.1 to only 8.4 mEq./l. In pa-

tient J. S. who was not severely acidotic, pulmonary ventilation was never above normal. He was therefore able to retain CO_2 , hence the pH and alkali reserve rose concomitantly. Similarly in patients T. H. and C. B., where sufficient time had elapsed between the two studies for adequate adjustment, both the pH and BHCO_3 had been brought to normal values. It is clear that determination of arterial or, less exactly, venous pH constitutes a more reliable and accurate picture of the acidosis and, by techniques now available, a more conveniently estimated measure than the usual plasma CO_2 combining power. With this safeguard, much of the objection which has been raised against the intravenous use of alkalis in the treatment of diabetic acidosis may be met. If it be conceded that acidosis in itself is not desirable there is little argument against the rapid correction of pH toward normal limits.

Arterial oxygen content and saturation. Many of these patients exhibited on admission higher values for arterial hemoglobin and oxygen content than is usual for hospital patients. This is most likely a reflection of associated dehydration and hemo-concentration. Even those patients whose admission values for blood hemoglobin concentration were not high were probably both dehydrated and anemic since determinations made after treatment invariably showed greater hemodilution. The figures for arterial oxygen saturation are approximations since oxygen capacity was not directly determined but was derived from the hemoglobin concentration and the factor 1.34 representing the oxygen capacity of one gram of hemoglobin. They indicate on the whole only a very slight reduction in arterial blood oxygen saturation (mean = 94%) which, in view of the experimental errors involved plus the generally severe acidosis which would be expected to depress the oxygen capacity of hemoglobin slightly, indicates the absence of significant interference with pulmonary gas exchange.

Pulmonary ventilation. The respiration was appreciably stimulated in practically all patients and, in many, was of the Küssmaul type. In an effort to ascertain the mechanisms possibly involved in this hyperpnea, the respiratory minute volume was tested for possible relationship with mean arterial blood pressure, arterial CO_2 tension, pH and ketone concentration, and rectal temperature.

There was little correlation except in the case of arterial pH, a graph of which is shown in Figure 1. This includes some data on patients not otherwise studied. The relationship between pulmonary ventilation and pH is somewhat complex. There is evidence of a threshold at about pH 7.20, respiration being relatively unaffected down to that point. Below pH 7.20 respiratory volume rises sharply to a maximum of 35 liters/min. in the region of pH 7.0. With increasing acidity the ventilation appears gradually to fall off. The initial threshold is similar to that of the carotid bodies to acidosis demonstrated by Comroe and Schmidt (21), and suggests that the mechanism for this hyperpnea may be a chemoreceptor reflex. That this stimulation should eventually give way to a depression as the acidosis increases in severity, possibly through depression of the medullary centers, is not unreasonable. It is interesting to note that in these patients, respiratory stimulation oc-

curs in the face of a profound fall in arterial CO_2 tension, indicating that at least in this condition the important regulatory function of carbon dioxide has been superseded by another agent, quite possibly hydrogen ion concentration. Our data for pulmonary ventilation and arterial pH after the acidosis has been corrected, although incomplete, suggest that the respiratory response lags considerably behind the response of pH to treatment, so that the hyperpnea may continue for a time after the arterial pH is close to normal. Should this observation be confirmed it would indicate either that the hydrogen ion is not the real factor concerned in the hyperpnea, or that some time must elapse before a change in the pH of arterial blood is reflected in the hydrogen ion concentration of the cells responsible for the respiratory stimulation.

Blood glucose and electrolytes. Values for blood glucose were elevated in all patients and indiscriminately with respect to the presence or absence of coma. This has also been the finding of others (1, 3, 5). The mean values for plasma electrolytes are shown graphically in Figure 2. Such studies have been made in some detail by others (22 to 24). These electrolyte patterns, in almost complete numerical agreement with those of Peters and associates (23), show a reduction in total base in diabetic acidosis, a considerable reduction in bicarbonate ion, and a progressive increase in the undetermined anion fraction (X) associated with, but by no means entirely explained by, an increase in total ketone bodies. These substances are abnormally elevated in the "acidosis" group and even more so in the patients with coma although the difference falls short of statistical significance. Thus, from the degree of acidosis, of hyperglycemia, of ketosis, and of disturbance in the blood electrolyte pattern there is little to explain the marked difference in mental state and prognosis between patients in diabetic coma and those with severe diabetic acidosis.

Mean arterial blood pressure. The average value for mean arterial blood pressure is definitely lower for the comatose patients (66 mm. Hg) than the normal figure of 86 mm. found in those who were acidotic but conscious, although this difference is short of statistical significance. This observation is explained by the well-established element of circulatory failure in diabetic coma most

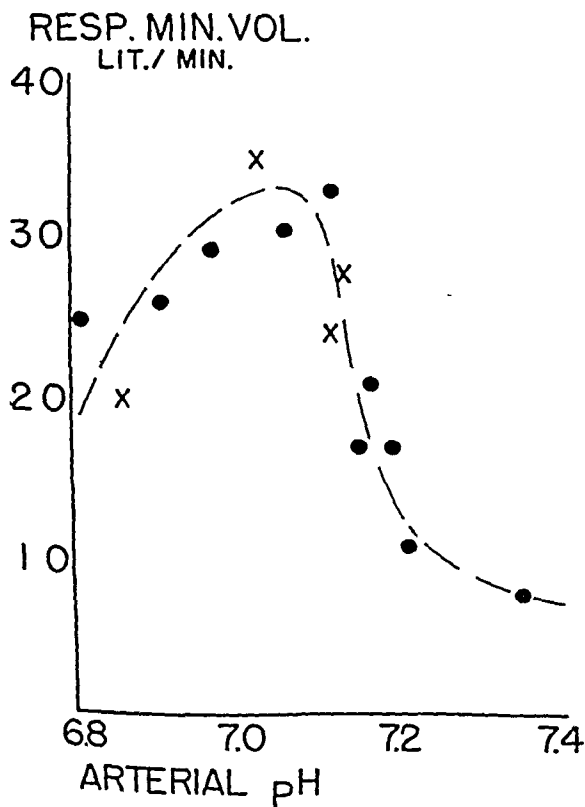


FIG. 1. THE RELATIONSHIP BETWEEN RESPIRATORY MINUTE VOLUME AND ARTERIAL pH

The circles represent patients who ultimately recovered, the crosses those who succumbed.

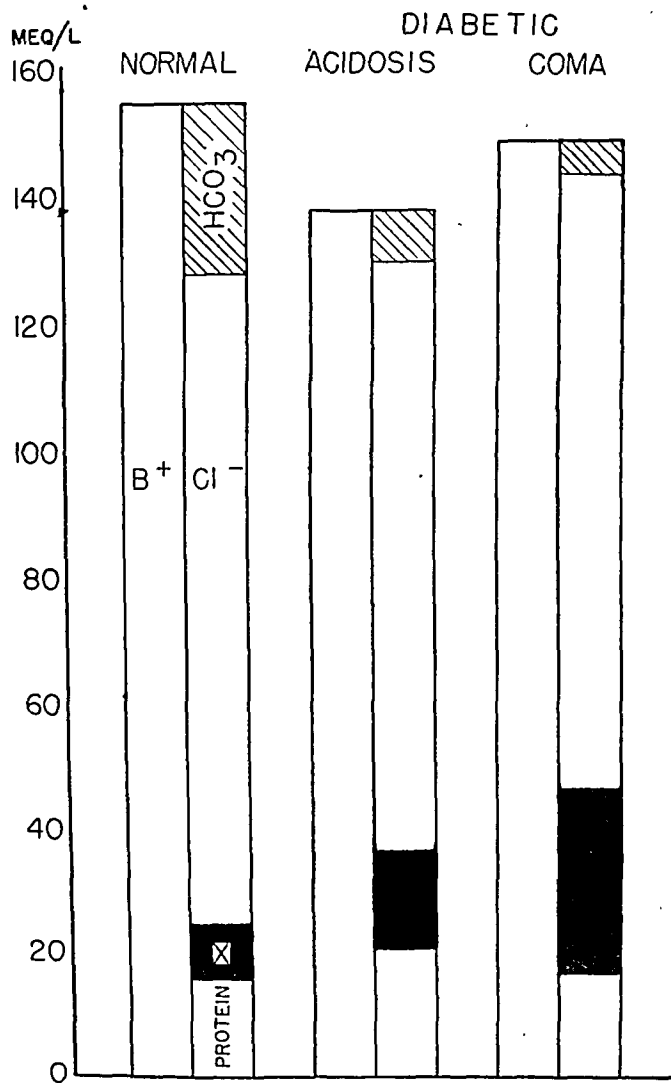


FIG. 2. THE BLOOD ELECTROLYTE PATTERN IN DIABETIC ACIDOSIS AND COMA

The anion fraction (X) represents phosphate, sulphate, and organic acids.

recently enunciated by Schechter, Wiesel and Cohn (25).

Cerebral blood flow and metabolism. In spite of the circulatory depression found in the comatose patients, only two showed a reduction in cerebral blood flow (CBF). The others yielded figures for this function somewhat in excess of the normal and the mean value for the group was 65 as compared with a normal figure of 54 cc./100 g./min. (12). The patients with acidosis displayed a greater consistency in cerebral blood flow which was slightly reduced on the average (45 cc./100 g./min.). This difference between the two groups was largely due to a difference in tone of cerebral vessels, patients in coma showing a fairly consistent decrease in cerebrovascular resistance

(CVR) (1.1 resistance units) while this function was slightly increased in those patients in acidosis without coma (2.1 units). The normal value for cerebrovascular resistance is 1.6 mm.Hg/cc./100 g./min. (12).

Some explanation for the surprising observation of an actual increase in cerebral blood flow in diabetic coma is suggested if the blood flow be plotted against the pH of arterial blood (Figure 3). There is a fairly good correlation ($r = -0.70$, $p = 0.01$) and the shape of the curve is similar to that found in normal subjects (26) where pH is altered by changing the pCO_2 of the blood. In the case of the diabetic acidosis group, however, the curve is shifted to the left so that for comparable levels of pH the blood flow is lower in these patients than in normal subjects breathing 5-7% carbon dioxide. This may be due to the fact that in the case of normal subjects breathing carbon dioxide both pCO_2 and hydrogen ion concentration are increased and their individual effects are likely to be summated, while in diabetic acidosis the in-

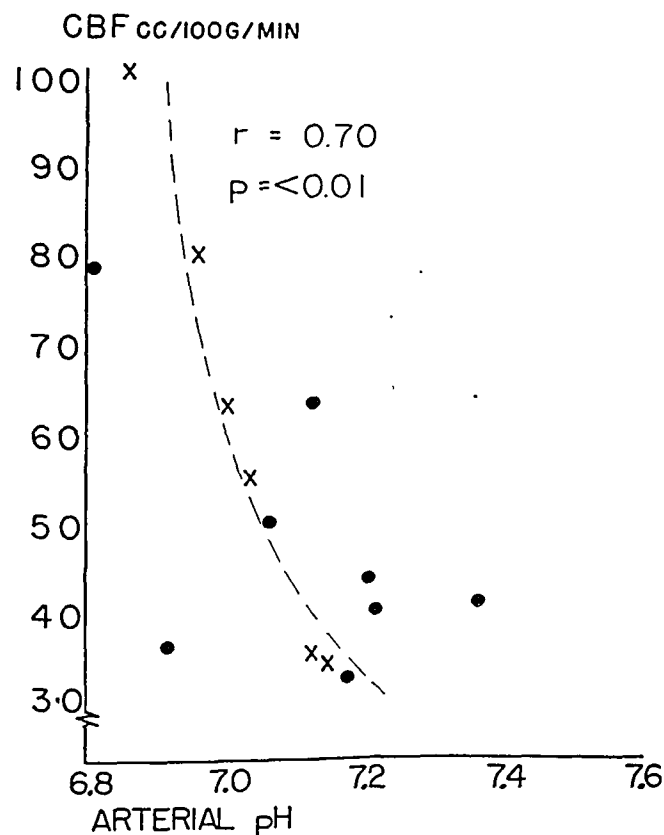


FIG. 3. THE RELATIONSHIP BETWEEN CEREBRAL BLOOD FLOW AND ARTERIAL pH

The circles represent patients who ultimately recovered, the crosses those who succumbed.

crease in hydrogen ion concentration occurs in the face of a marked fall in $p\text{CO}_2$. It is not yet clear in the case of CO_2 inhalation whether the cerebrovascular dilation is due to the CO_2 itself or to the concomitant pH shift or to both; it is nevertheless evident that at least in the severe acidosis of diabetes, cerebral vessels may dilate even though the CO_2 tension be remarkably low.

Of all the studies performed, measurement of cerebral metabolic rate in terms of oxygen consumed (CMR_{O_2}) yielded by far the most significant difference between the comatose and non-comatose patients. As compared with a consumption of 3.3 ($\sigma = \pm 0.4$) cc. of oxygen per 100 g. of brain per minute found in mentally alert normal subjects (12), the comatose patients yielded an average of only 1.7 ($\sigma = \pm 0.4$), a reduction of 48% and highly significant statistically. Those patients who were acidotic and confused but not unconscious on admission exhibited an average figure for cerebral O_2 consumption of 2.7 ($\sigma = \pm 0.4$) cc./100 g./min., moderately lower than the normal. In fact there seems to be a critical level for cerebral oxygen utilization of 2.1 cc./100 g./min. at or below which consciousness disappears.

The cerebral respiratory quotient was consistently below unity, averaging 0.87 and 0.92 in the comatose and non-comatose groups, respectively. At the present state of our knowledge there is little justification for speculation on the basis for this slight but significant deviation from the normal of 0.99 (12).

It was hoped that by determination of cerebral arteriovenous glucose difference the utilization of this substance by the brain could be measured in this condition as has already been done in hypoglycemia (8). At the high blood sugar levels encountered, however, the arteriovenous difference was well within the error of the glucose determinations so that such an estimation is not possible until blood glucose methods of much higher precision are available.

DISCUSSION

It has been a discouraging finding repeatedly confirmed that the usual biochemical analyses in patients with severe diabetic acidosis fail to show any significant difference between those who are

conscious, whose metabolic derangements are readily reversible by present therapy, and those who are in true coma with a grave prognosis, and a frequently irreversible pattern of deterioration. The present studies indicate that cerebral oxygen utilization is at least one biochemical function which serves to differentiate these two groups. In all of the patients who were in coma on admission the cerebral oxygen consumption was 2.1 cc./100 g./min. or less while seven of the eight patients who still retained consciousness yielded a value for this function of 2.4 cc./100 g./min. or more. This measurement was also the only one of the many performed which had definite prognostic significance. With but one exception in each group, a cerebral oxygen consumption below 2.1 cc./100 g./min. was incompatible with survival, whereas in those with values above that figure response to treatment was satisfactory and recovery took place. This depression in oxygen utilization is not limited to the brain. Schechter, Wiesel and Cohn have demonstrated a decrease in oxygen consumption in the extremities of patients in diabetic acidosis (25). In the brain, however, a defect in metabolism is of grave and immediate significance. The interesting correlation between mental state and utilization of oxygen by the brain is shown in Figure 4. The reduction in cerebral metabolism is accompanied by and undoubtedly

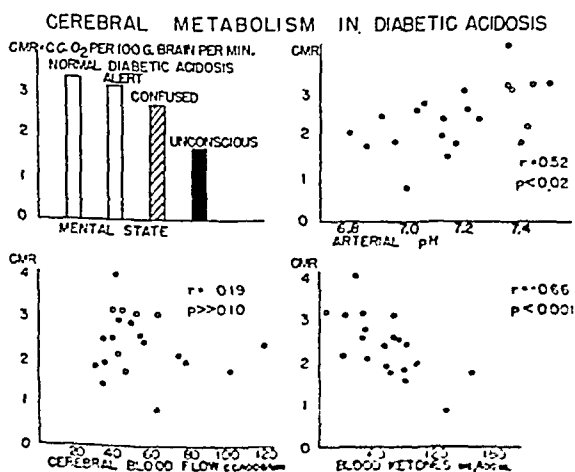


FIG. 4. CORRELATIONS OF CEREBRAL OXYGEN CONSUMPTION (CMR) WITH MENTAL STATE, CEREBRAL BLOOD FLOW, ARTERIAL pH AND ARTERIAL KETONE CONCENTRATION

The closed circles represent admission data, the open circles observations made in the course of therapy.

responsible for the progressive deterioration of mental function which occurs in the course of uncorrected diabetic acidosis. We have observed a strikingly similar phenomenon in the hypoglycemia and coma induced by insulin (8), the stage of severe hypoglycemia and confusion being associated with a CMR_{O_2} of 2.6 and the state of deep coma yielding an average of 1.9 cc. O_2 /100 g./min. Whereas in insulin hypoglycemia the depression of cerebral metabolism may reasonably be attributed to the profound drop in glucose available for utilization by the brain, it is by no means as easy to identify the process responsible for the decreased cerebral oxygen utilization in diabetic coma. There is evidence to indicate that insulin from the pancreas may be dispensable in the utilization of glucose by the brain (9), a very cogent observation being that in untreated human diabetics and depancreatized animals mental function is interfered with only late in the progress of the acidosis and other biochemical derangements. In a search for the possible factors directly responsible for this depressed utilization of oxygen by the brain, it may be pertinent to determine the correlation between CMR_{O_2} and each of the biochemical and physiological disturbances known to occur in diabetic coma. On the basis of the circulatory failure usually found in this condition the reasonable thesis has been proposed that the coma is due to a deficiency in cerebral circulation (27). Our findings demonstrate that this is not so (Table III), and a correlation between CMR_{O_2} and CBF (Figure 4) indicates that the cerebral oxygen utilization in this condition is almost completely unrelated to the cerebral blood flow. On the basis of this fact and the relatively normal arterial oxygen saturations observed, it may be concluded that the fault in cerebral oxygen utilization does not lie in the supply of oxygen to the brain. The possibility that the mechanisms for release of oxygen from the blood to cerebral tissue may be disturbed is now under investigation by a study of the oxyhemoglobin dissociation curve and the oxygen tensions in cerebral venous blood. With the reservation that a significant defect may be found there, it is probable that the fundamental derangement is in the cellular biochemical processes responsible for the normal utilization of oxygen. If these processes are at all dependent on the environmental pH, and this can hardly be

doubted, the acidosis itself might be expected to contribute to the depression in cerebral metabolism. There is a fair correlation (Figure 4) between CMR_{O_2} and arterial pH which tends to justify this supposition. Of course the pH of arterial blood only indirectly affects the hydrogen ion concentration inside the cell and it is possible that this correlation would be considerably improved were the latter quantity measurable.

In 1914 Hurler (28) suggested that acetoacetic acid was a noxious agent in diabetic acidosis and despite conflicting evidence (29), recent work has corroborated the toxic properties of this substance. Schneider and Droller (30) found that slow intravenous infusion of acetoacetic acid in rabbits regularly produced coma where even a greater acidosis resulting from administration of hydrochloric or beta hydroxybutyric acids had no such effect. In fact coma was consistently obtained even with sodium acetoacetate where no acidosis accompanied the administration. Thus at least one of the ketone bodies is capable of producing coma although the blood concentrations necessary are not known and may well be higher than those found in the coma of diabetic acidosis. Our own observations are compatible with the thesis that ketosis is an important factor contributing to this type of coma. Figure 4 shows a fairly good correlation between CMR_{O_2} and blood ketone concentrations. Cerebral oxygen utilization fell as the blood ketone level rose. Such a correlation is open to a number of interpretations. It may mean that one or more of the ketone substances, acting as a histotoxic agent, is responsible for the depression in cerebral metabolism. An equally good possibility is that the blood ketone level is simply an index of less defined but more fundamental aberrations just as the blood urea concentration reflects, but is hardly responsible for, the disturbances in uremia. There is more to be said for the causal efficacy of the ketones, however, in that at least one of their number has been shown to be capable of producing coma in itself.

The results reported here are compatible with, but by no means demand, the following sequence of events. The glycosuria plus the loss of sodium from the body lead to the well-recognized but inadequately verified contraction in extracellular fluid space of which the blood volume is an im-

portant component (31, 32). This can only result in a decrease in cardiac output compensated by a restriction in peripheral blood flow (25) and quite probably a marked decrease in renal blood flow. The work of McCance and Lawrence (33) as well as that of Peters, Kydd, Eisenman and Hald (23) stresses the importance of renal regulatory mechanisms in the excretion of keto-acids, while the experiments of Stadie, Zapp and Lukens (34) demonstrate the major role which peripheral tissues play in the utilization of ketone bodies. Thus this diversion of the decreased cardiac output from the kidneys and muscles, although necessary for the maintenance of blood flow through more immediately vital centers, sharply restricts the available mechanisms for the utilization and excretion of ketone bodies produced in this condition in excessive amounts (35). The important renal adjustment of the body hydrogen ion concentration is also disorganized (33). The resultant acidosis and ketosis, not to mention a number of poorly defined but possibly more important biochemical disturbances, produce serious derangements in cellular oxidations throughout the body. In the heart these derangements may lead to further circulatory failure, now on the basis of myocardial inefficiency in addition to the decreased blood volume. These disturbances in metabolism in the brain are probably responsible for the development of coma and eventual death.

The nature of the "irreversibility" of severe diabetic coma is somewhat indicated by the present studies but by no means clearly defined. It is one thing to establish a critical level of cerebral oxygen consumption below which death occurs in spite of therapy, but quite another to explain the nature of the process which cannot be reversed. One can only hope that further study will lead to a deeper insight into these processes for certainly the irreversible stage in this disease is relative only to our ability to comprehend and correct the biochemical and physiological aberrations which comprise it.

SUMMARY

1. Studies of blood gases, electrolytes, acid-base balance, respiration, blood pressure, cerebral blood flow and cerebral oxygen consumption are reported on 14 patients in severe diabetic acidosis, six of whom were in deep coma.

2. Respiratory minute volume in these patients was well correlated with arterial pH.

3. Coma was associated with and probably the result of a 40% reduction in cerebral utilization of oxygen which occurred in spite of a generally augmented cerebral blood flow and a normal arterial oxygen saturation.

4. The depression in cerebral oxygen consumption is partly related to the acidosis and more significantly to the ketosis in this condition although other factors as yet poorly defined are undoubtedly operating.

5. The results establish the feasibility of applying these techniques in diabetic coma and open the possibility of further definition of the biochemical derangements in the living human brain by the study of more specific metabolic components.

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THE BLOOD FLOW, VASCULAR RESISTANCE, AND OXYGEN CONSUMPTION OF THE BRAIN IN ESSENTIAL HYPERTENSION ¹

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The vascular bed of the brain should occupy a prominent place among the regions of interest to the clinical investigator who would seek to define the circulatory disturbances associated with hypertension. The vulnerability of cerebral arteries in chronic hypertensive disease has long been recognized. Statistical surveys demonstrate that about 15% of deaths in hypertension are the result of cerebrovascular lesions (1 to 3). Many hypertensive patients experience symptoms such as headache, dizziness, and tinnitus, which are probably referable to the brain. Furthermore it has been suggested that some of the etiological factors in hypertension may be of cerebral origin.

Knowledge of the cerebrovascular resistance and cerebral blood flow in human hypertension has been extremely limited. The well-recognized changes observed in retinal vessels, in the absence of more specific information, have been presumed to reflect similar phenomena in the cerebral vascular bed. Measurement of cerebral blood flow in this disease has been limited to studies of cerebral arteriovenous oxygen differences (4, 5) which become an index of blood flow only after cerebral metabolism can be measured or shown to be normal.

The nitrous oxide method for the quantitative measurement of human cerebral blood flow (6, 7) found its first application to clinical disease in a study on five patients with various degrees of arterial hypertension (8). The present study represents a more complete investigation on 13 additional patients.

METHODS

Patients in whom nearly uncomplicated essential hypertension existed were selected from the medical or surgical services. Cerebral blood flow (CBF) was determined by the nitrous oxide method (7), with the use of a gas mixture consisting of 15% N₂O, 64% N₂, 21% O₂. Mean

¹ The expenses of these studies were defrayed by a grant from the Life Insurance Medical Research Fund.

arterial blood pressure (MABP) was obtained by means of a damped mercury manometer attached to a needle in the femoral artery. Cerebral metabolic rate in terms of cerebral oxygen consumption (CMR_{O₂}), and cerebrovascular resistance (CVR) were calculated as previously described (7). Blood gas analyses were made with the Van Slyke-Neill manometric apparatus (9). Potentiometric measurement of blood pH was made anaerobically at 37° C. by means of a glass electrode. Values for blood carbon dioxide tension were calculated by means of the nomograms presented by Peters and Van Slyke (9).

RESULTS

The data obtained in these measurements are presented in Table I. There is little deviation from the normal in most of the values found. There is an even distribution between the sexes, but most of the patients lie in one age group—the fifth decade of life. It should be pointed out that the normal values presented for comparison were obtained in a series of young men in the third decade (7). We have observed no measurable trend in any of our data to suggest that normal values in middle-aged individuals differ significantly from those in somewhat younger subjects.

The mean arterial blood pressures in this series range from 124 to 190 mm. of mercury, the average value of 159 mm. being 89% above the normal of 86 mm. Values for the carbon dioxide content and tension, pH, and oxygen content of arterial and internal jugular blood are within normal limits.

Cerebral blood flow, cerebral oxygen consumption, and cerebral arteriovenous oxygen difference are all practically identical with mean normal values. The cerebral respiratory quotient

$$\left(\frac{\text{cc. CO}_2 \text{ produced}}{\text{cc. O}_2 \text{ consumed}} \right) \text{ by the brain}$$

is slightly but significantly depressed below the average normal value of 0.92. The most striking deviation from the normal observed in the entire study is the marked and consistent elevation of

TABLE I
Blood gases and cerebral circulation and metabolism in essential hypertension

Pt.	Age yrs.	Sex	Color	Reti- no- pathy grade	Arterial					Internal jugular					Cerebral			
					B. P.	CO ₂ con- tent	CO ₂ ten- sion	pH	O ₂ con- tent	CO ₂ con- tent	CO ₂ ten- sion	pH	O ₂ con- tent	A-V O ₂	CBF	CMR O ₂	CVR	RQ
					mm. Hg	vol. %	mm. Hg		vol. %	vol. %	mm. Hg		vol. %	vol. %	cc./100 g./min.	cc./100 g./min.	mm. Hg cc./100 g./min.	
M. O.	40	F	W	IV	190	44.2	31	7.50	17.5	49.5	37	7.45	10.5	7.0	54	3.8	3.5	0.77
F. H.	54	F	W	II	124	50.8	43	7.40	17.6	57.5	50	7.37	10.9	6.7	56	3.8	2.2	1.00
B. P.	37	F	W	II	159	41.2	35	7.40	18.3	47.4	42	7.37	12.8	5.5	63	3.5	2.5	1.13
G. J.	48	M	C	III	161	47.5			17.8	53.8			11.2	6.6	52	3.4	3.1	0.95
W. M.	47	F	C	II	155	50.1	40	7.42	16.2	57.3	51	7.36	8.7	7.6	47	3.5	3.3	0.95
J. M.	49	M	C	III	166	56.8	45	7.42	15.8	62.1	56	7.35	8.5	7.3	45	3.3	3.7	0.73
R. O.	34	M	W	III	158	56.3	48	7.39	16.4	61.7	62	7.31	11.2	5.2	52	2.7	3.0	1.04
L. S.	48	M	W	II	171	52.3	43	7.41	17.7	57.1	52	7.36	12.8	4.9	64	3.1	2.7	0.98
H. K.	38	F	W	IV	190	49.8	41	7.41	16.6	55.5	50	7.36	10.1	6.5	59	3.8	3.2	0.88
M. Mc.	47	F	W	I	175	47.6	42	7.37	15.9	52.6	50	7.34	9.2	6.7	52	3.5	3.4	0.75
L. T.	46	M	C	II	137	49.9	46	7.36	17.6	55.4	56	7.31	11.4	6.2	55	3.4	2.5	0.89
L. R.	47	M	W	II	157	47.6	41	7.39	18.1	52.8	52	7.32	12.3	5.8	54	3.1	2.9	0.90
N. B.	51	F	W	III	126	49.9			16.1	54.9			9.8	6.2	44	2.7	2.9	0.79
Mean	45				159*	49.5	41	7.41	17.0	55.2	51	7.36	10.7	6.3	54	3.4	3.0*	0.90*
Mean values in normal young males					86	49.4	42	7.40	17.3	55.4	51	7.34	11.0	6.3	54	3.3	1.6	0.99

* Signifies statistically significant differences from the normal.

cerebrovascular resistance, a measure of the tone of cerebral vessels obtained by dividing the cerebral blood flow into the mean arterial blood pressure (7). Whereas in the normal brain a pressure head of 1.6 mm. of mercury is necessary to produce a flow of 1 cc. of blood per 100 grams of brain per minute, a pressure of 3.0 mm. or nearly twice the normal produces the same flow through the brain of hypertensive patients.

DISCUSSION

Observers who have made clinical measurements of blood flow in various organs of hypertensive patients have found a relatively normal volume flow of blood in the face of a considerably elevated perfusion pressure in the arterial system. In the body as a whole, total blood flow (*i.e.*, cardiac output) has been found to be relatively normal in hypertension by Bradley and Smith (10) who used the ballistocardiograph, and Goldring, Chasis, Ranges, Lauson, and Cournand (11) who employed the direct Fick principle. Skin temperature measurements (13) and observations with the occlusion plethysmograph (14, 15) indicate a normal peripheral blood flow in this disease. The studies of Goldring, Chasis, Ranges and Smith (16) on renal blood flow in hypertensive patients

indicate that renal blood flow per mass of functional excretory tissue is reduced very slightly, if at all. Work recently reported by Wilkins and collaborators (17) demonstrates that blood flow through the abdominal viscera is not altered by the presence of hypertension.

To this list of organs in which clinical blood flow measurements have been made in hypertension the brain may now be added. Our results in the present series and its precursor (8) demonstrate for the first time that cerebral blood flow is within normal limits in patients with uncomplicated essential hypertension. The mean value of 54 cc./100 g./min. presented in Table I is identical with the mean for normal subjects (7). The average of the five cases in the preliminary report (after correction of the brain/blood partition coefficient for nitrous oxide from the earlier estimate of 1.3 to the more exact value of 1.0 [18]) was 49 cc./100 g./min., representing a difference from the present study which is not statistically significant.

A normal cerebral blood flow in the presence of a high mean arterial blood pressure can only mean an increase in the resistance to the flow of blood offered by the cerebral vascular bed. Calculation of this cerebrovascular resistance in these

patients shows an increase over the normal of 88%. It is further apparent that if the blood flow in other vascular beds such as the kidney, the abdominal viscera, extremities, skin and the total cardiac output are not increased in this condition, as indeed all the experimental evidence previously cited demonstrates, then an increase in vascular resistance of at least equal magnitude must occur in each of these circulatory beds and in the body as a whole. Thus the brain shares in equal measure the generalized increase in vascular tone which is associated with hypertension.

The cerebral arteriovenous oxygen difference in this series yielded a mean value (6.3 vol. %) identical with that found by us in normal subjects (7). This is in accord with the findings of Williams and Lennox (4) who obtained a normal cerebral arteriovenous oxygen difference on 21 hypertensive patients. Raab (5), on the other hand, reported a mean value of 7.7 vol. % in ten patients with this disease and concluded that relative medullary ischemia may have been responsible for the hypertension. A possible explanation for this discrepancy may be the greater age and the more advanced state of the hypertension in the patients studied by Raab. Our finding of a normal cerebral blood flow and normal hydrogen ion, CO_2 and oxygen concentrations in the internal jugular blood of a younger group of hypertensives speaks against cerebral ischemia as a primary factor in essential hypertension.

Examination of Table I reveals some correlation between the cerebrovascular resistance and the grade of retinopathy even though the four grades are not evenly represented. If these 13 patients plus three normals and the five hypertensives previously reported are ranged in order of increasing severity of retinal vascular changes and of increasing cerebrovascular resistance, a coefficient of correlation may be obtained with a high order of significance ($r = 0.66$, $p = 0.001$). Thus the clinical impression that the retinal vessels reflect the state of the vessels of the brain appears to be substantiated by these quantitative observations. A future report on a larger and more representative series will concern itself specifically with this aspect of the subject.

The present extent of our data permits no conclusion as to the sequence of events between

the hypertension and the cerebrovascular hyper-tonus. The classic studies of Cushing (19), recently confirmed by us in man (20), demonstrate that restriction of cerebral blood flow may result in a compensatory increase in blood pressure. Perhaps similar phenomena are the hypertensive states experimentally produced by Dixon and Heller (21) as the result of intracisternal injection of kaolin, or by Novak and Walker (22) with serial ligation of the arterial supply to the brain. There is thus at least some evidence to favor the hypothesis that in essential hypertension there may be a primary cerebrovascular constriction accompanied by a secondary and compensatory hypertension which maintains a normal cerebral blood flow. If this be the primary mechanism, however, its *modus operandi* can hardly be through cerebral asphyxia, since there is no detectable change in the chemical composition of internal jugular blood. There is equal plausibility, however, in two alternative concepts: that the increased cerebrovascular resistance is simply part of a generalized vasoconstriction which raises the blood pressure, or that it occurs in response to a hypertension originating elsewhere and is mediated by a hypothetical intrinsic regulating mechanism, the effect of which is to maintain a normal cerebral blood flow.

It is possible that further investigations, now under way, of the cerebral circulation in hypertension, especially in its response to alterations in the level of arterial blood pressure, or to block of the cranial sympathetic supply, may shed further light on the relationships between hypertension and the marked increase in cerebrovascular resistance which accompanies it.

SUMMARY

1. Studies are reported of cerebral blood flow, cerebral metabolism and cerebrovascular resistance in 13 patients with essential hypertension.
2. Despite a markedly elevated mean arterial blood pressure, cerebral blood flow was within normal limits, as was also cerebral oxygen consumption.
3. There was a marked and consistent increase in cerebrovascular resistance averaging 88%, which appeared to be roughly correlated with the grade of retinopathy.

ACKNOWLEDGMENT

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THE URINARY EXCRETION OF INSULIN BY NORMAL AND DIABETIC SUBJECTS ^{1,2}

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The fate of insulin injected into the blood stream is unknown. During the course of studies designed to investigate this problem, it became pertinent to inquire into the excretion of insulin from the body. Previous investigations on the urinary excretion of insulin have led to conflicting reports. Some studies (1, 2) have indicated that normal urine is capable of eliciting an hypoglycemic response which has been interpreted as proof of the presence of insulin in such urine. Other investigators (3, 4) have reported their inability to detect appreciable amounts of this hormone in the urine of man. In view of the controversial nature of such results and the need for more knowledge concerning the fate of injected insulin, it appeared advantageous to reinvestigate the problem of the urinary excretion of insulin.

The controversial results reported in the literature may be attributed partly to the technical difficulties involved in the estimation of a low concentration of insulin in a fluid menstruum such as urine. If urine contains very small quantities of insulin, then the equivalent of large volumes of urine would be required to produce a reliable hypoglycemic response. However the complex nature of urine and the presence of toxic substances may mask any hypoglycemic effect of the contained insulin by virtue of a toxic glycogenolytic effect. Accordingly the injection of crude urine cannot be utilized as a reliable procedure for the assay of insulin.

Cutting (4) attempted to circumvent this difficulty by precipitation of the insulin from urine with ammonium sulfate. She demonstrated that this procedure could be used effectively to recover 0.2 units of insulin from 100 ml. of urine but

could not detect the presence of insulin in normal human urine with this procedure. Consequently she concluded that human urine must contain less than 0.2 units per 100 ml. If a normal individual excretes in the neighborhood of 1400 ml. of urine per day, such results would imply that less than 2.8 units of insulin are excreted each day. In order to detect such small quantities of insulin, it became necessary to devise a method adequate for this purpose. Accordingly, we have adopted for the estimation of the insulin content of human urine the following procedure which represents a modification of a method used in this laboratory for the preparation of urines for the extraction of other protein hormones excreted through the kidney.

METHODS

Specimens of urine were collected from each subject for periods varying from 24 to 120 hours. Every voided specimen was placed immediately in the refrigerator and kept in the cold until an entire day's collection had been obtained. Previous studies (4), confirmed by us, demonstrated no appreciable inactivation of insulin during this procedure. In order to remove the urinary salts, the 24-hour specimens were dialyzed overnight, against running ice cold water, in a Visking dialysis membrane. The dialyzed fluids were immediately shell frozen and desiccated by the lyophile process. The small amounts of dry powder obtained in this manner could be combined with similar dried products from the collection of urine on other days. The powders resulting from this procedure were fluffy, non-hygroscopic, easily handled and could be stored indefinitely without deterioration. If the urinary salts were not removed by dialysis prior to lyophilization, the resulting powders were hygroscopic, could be handled only with difficulty and could not be preserved in a stable form.

The average daily urine output of normal subjects was thus concentrated to a small quantity of the final dry product. The insulin in this small amount of solid material could be extracted by the same procedure as that which has been utilized for the assay of the insulin content of pancreatic tissue. Ten ml. of 70 per cent alcohol plus 0.03 ml. of concentrated sulfuric acid were added to

¹ Aided by grants from the New York Diabetes Association and the Eli Lilly Company.

² Presented in part before the American Physiological Society, Atlantic City, March, 1948.

each gram of powder. The mixture was stirred in a Waring Blender for five to ten minutes and centrifuged immediately. The residual undissolved material was re-extracted in the same manner. The combined extracts were then concentrated *in vacuo* in order to reduce the volume, care being taken to insure that the acid concentration was not allowed to exceed 3 per cent during this process. Alcohol was added to the concentrated extract to a final concentration of 92 per cent. This was followed by the addition of 1.5 times this final volume of ethyl ether. The mixture was chilled and allowed to stand overnight in the cold room. The clear supernatant fluid was removed by decantation the following morning. The remaining precipitate was taken up in acid water and the residual traces of alcohol and ether removed *in vacuo*. The final acid water extract was made up to a definite volume and its insulin content determined immediately by the Young and Lewis (5) modification of the mouse assay.

For reasons which will be discussed below, it was found most feasible to perform urine insulin assays on specimens voided during 72 or more hours of collection. The analysis of such specimens revealed amounts of insulin ranging from 0 to 1.2 units; the mean quantity of insulin determined in such specimens being 0.48 units. It therefore appeared probable that a method capable of detecting the presence of from 0.4 to 0.5 units of insulin in urine could be considered valid for the purpose of this investigation.

To test the validity of the method of extraction and assay, from 0.4 to 0.5 units of insulin were added to urine which was then subjected to the entire process outlined above. A number of experiments demonstrated that the procedure detects small amounts of insulin. The results in Table I demonstrate recovery of from 60 to 100 per cent of 0.4 units of insulin added to the lyophilized powder obtained after dialysis of large volumes of urine. Furthermore, the addition of similar small amounts of insulin to urine prior to its dialysis revealed no appreciable loss of insulin during the process of dialysis. The results in Table I can be taken as indicative of the degree of the quantitative accuracy of our procedure for the determination of those amounts of insulin which were found to be excreted in urine. The results in this table also demon-

TABLE I

Recovery of insulin added to crude and lyophilized urine

Volume of crude urine	Weight of lyophilized urine powder	Quantity of insulin added	Quantity of insulin determined	Recovery of added insulin
ml.	grams	units	units	per cent
1,000		0	0.12	
1,000		0.5	0.43	64
	2	0	0.08	
	2	0.5	0.38	60
	0.83	0	0.13	
	0.83	0.4	0.58	112

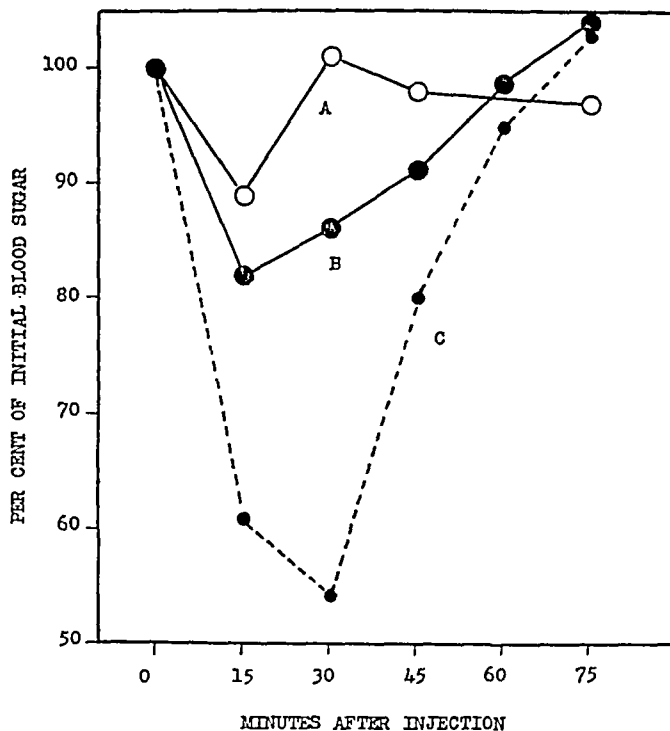


FIG. 1. EFFECT OF URINE EXTRACTS ON THE BLOOD SUGAR OF THE RABBIT

- A—Extract from 3 liters urine.
- B—Extract from 5 grams urine powder.
- C—Extract from 5 grams same urine powder to which 1 unit insulin had been added.

strate the fact that this method of analysis could detect the presence of the very small but appreciable amount of insulin excreted by normal subjects.

Although results obtained with the mouse convulsion assay and its various modifications are considered by convention specifically to detect the presence of insulin and although such assays have frequently been utilized to measure the insulin content of various organs and tissues, it is conceivable that the fall of the mice in the Young and Lewis assay might be due to factors other than insulin in the final acid saline extract. It therefore appeared advantageous to demonstrate that such extracts of urine actually contain a substance capable of producing a drop in the blood sugar of the rabbit. This was accomplished by the intravenous injection of the urine extracts into fasted rabbits and the determination of the degree of hypoglycemia produced. A typical protocol of such a procedure is found in Figure 1, which demonstrates that urine extracts actually do contain a substance which produces a fall in the blood sugar of rabbits.

Any substance of physiological origin which produces a transient hypoglycemia may be considered to be insulin. Furthermore, the degree of hypoglycemia produced in the rabbits corresponds roughly to the amount of insulin in such extracts as determined by the Young and Lewis mouse assay. Carefully controlled experiments of Cutting (4) demonstrated that a drop in the blood sugar of the rabbit of 25 mg. per cent corresponds to the presence of 0.2 units of insulin.

Further proof of the similarity of the substance in urine extracts, responsible for the fall of mice in the Young and Lewis assay, and insulin was obtained by a comparison of the behavior of the two substances to heat. The results in Table II demonstrate that heating in a boiling water bath for one hour resulted in the abolition of the ability of both aqueous solutions of insulin and urine extracts to produce falls in mice in the Young and Lewis assay.

TABLE II
*The effect of heating on the activity of
urine extracts and insulin*

Urine extract assayed	Insulin added	Exposure to heat	Units insulin determined by assay
A	No	None	0.4
A'	No	100° C., 1 hr.	0.1
B	Yes	None	0.7
B'	Yes	100° C., 1 hr.	0.1

An acid saline extract was prepared from 4 grams of lyophilized urine powder as described in the text. The final extract was divided in four aliquots of 7.5 ml. each. 2.5 ml. of acid saline were added to each of two aliquots (A and A'). 2.5 ml. of a solution of insulin in acid saline containing 16U/100 ml. were added to each of the other two aliquots (B and B'). A' and B' were immersed for one hour in a boiling water bath and then cooled to room temperature and each restored to its original volume of 10 ml. The insulin content of each of the four solutions was then measured by the Young and Lewis mouse assay.

The results of these experiments, namely, the recovery of insulin added to urine before and after lyophilization, the hypoglycemic activity of urine extracts and their resemblance to insulin in regard to heat inactivation are believed to constitute strong presumptive evidence that the Young and Lewis assay actually detects and measures quantitatively the amount of insulin excreted in human urine.

RESULTS

Numerous assays of samples of urine collected for 24-hour periods revealed that normal subjects excrete minute but detectable amounts of insulin. However, in order to permit a comparison between individuals and between groups, it was deemed essential to prepare for assay extracts containing such amounts of insulin as would tend to increase the accuracy of the analytical procedure. Consequently, extracts were prepared from the combined lyophilized products of urine collections obtained over periods of from 72 to 120 hours. The data obtained from such assays are presented in Figure 2 in which the absolute amount of insulin detected is expressed in terms of units excreted per 24 hours. The average daily insulin excretion of a group of 11 non-diabetic subjects was found to be 0.16 units with a

standard deviation of ± 0.04 units, with extremes of 0.10 to 0.24 units per day.

The insulin detected in the urine of these healthy subjects must have originated from endogenous sources since they received no exogenous supply. Therefore it appears obvious that only minute amounts of the insulin excreted by the pancreas ever escapes destruction (or utilization) within the body. It is conceivable that the rate of this insulin destruction might, in the healthy subject, just balance the rate of its pancreatic secretion. If such were the case, an exogenous supply of insulin might escape destruction and be excreted in the urine. In order to test this hypothesis several non-diabetic patients who were being subjected to shock therapy were given large amounts of insulin intravenously and the urine was collected at intervals thereafter and assayed for its insulin content. In no instance did this result in the recovery of more than infinitesimal fractions of the amount injected. Thus, in one instance, 400 units were given intravenously. From the urine voided during the first hour after injection 0.22 units were recovered, whereas 0.59 units were found in the urine excreted in the entire 24 hours after injection. This last quantity represents an increase of 0.43 units in excess of the mean daily excretion of the normal subjects considered in Figure 2. According to this calculation, only about 0.1 per cent of the amount of insulin injected was excreted within 24 hours. Such results have been typical of our failure to detect appreciable quantities of either endogenous or exogenous insulin in the urine.

In view of the fact that it is generally assumed that diabetic subjects suffer from an insulin insufficiency, it became pertinent to compare the insulin excretion of the diabetic patient with that of the apparently healthy subject. Although it would have been preferable to have utilized severely diabetic subjects for this purpose, such was not considered feasible since this would have necessitated the deprivation of insulin from such patients for 36 or more hours. Consequently, we studied the urinary excretion by a group of seven subjects whose metabolic disturbances were of such a degree of mildness that they did not require insulin for the regulation of their disease. The data obtained from these subjects are illustrated in Figure 2 which reveals that the average daily excretion of

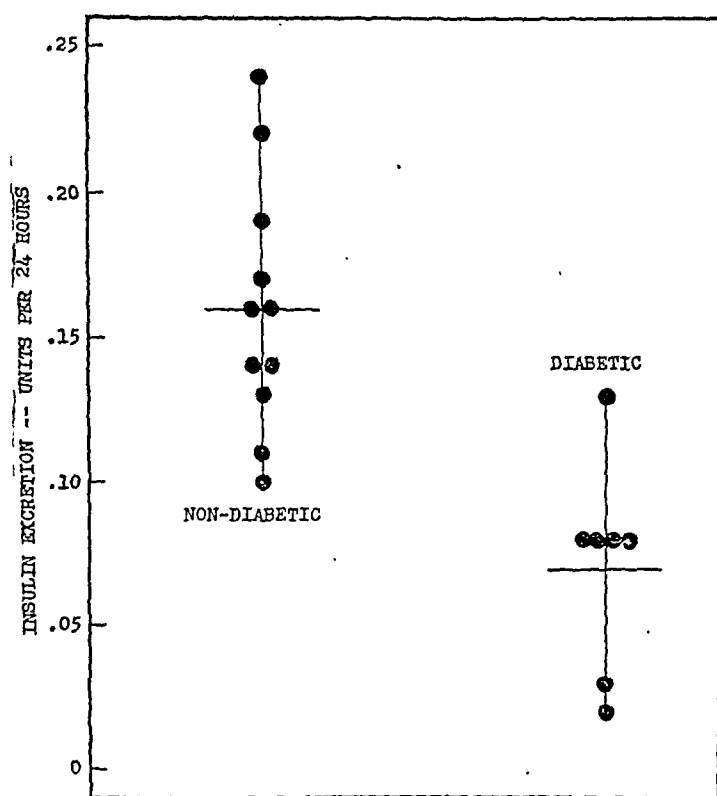


FIG. 2. URINARY INSULIN EXCRETION BY NON-DIABETIC AND DIABETIC SUBJECTS

The black circles indicate the excretion values of individual subjects. The horizontal lines indicate the mean insulin excretion per day for the respective groups.

these diabetic patients was 0.07 units with a standard deviation of 0.03 units, with extreme ranges of 0.02 and 0.13 units per day.

When urine was collected from a group of diabetic patients receiving insulin in quantities sufficient to control their metabolic disturbances, their insulin excretion per day was found to correspond to that observed in the non-diabetic subjects. Two diabetic patients with so-called "insulin resistance" were also studied. They excreted less insulin than did non-diabetic subjects to whom large amounts of exogenous insulin were administered. Thus, a 14 year old girl who required 240 units daily for the regulation of her diabetes excreted 0.2 units per day. Another patient, who required 350 units per day, excreted 0.3 units per 24 hours. Such values were significantly lower than the 0.6 units excreted after the injection of 400 units into the non-diabetic subject.

DISCUSSION

It has been postulated by many clinicians that the administration of insulin in multiple divided doses might be more economical than the adminis-

tration of a similar amount in a single dose. This claim has been based on the assumption that an appreciable fraction of the single dose of insulin might be excreted more rapidly than it could be used within the body. Our results fail to substantiate such an assumption since we find that only infinitesimal fractions of even tremendous doses of insulin are ever excreted in the urine. Presumably, therefore, wastage of insulin as a result of its excretion is of no practical consideration.

The average daily excretion of normal subjects was found to be 0.16 ± 0.04 units per day; the average excretion of patients with diabetes mellitus was found to be 0.07 ± 0.03 . The difference between the means of the two groups is 5.6 times greater than the standard error of their difference, thus indicating a statistically significant difference in the insulin excretion of the two groups. In view of the very small difference in the absolute amount of insulin excreted by the two groups, it cannot be stated conclusively that the average mild diabetic patient is characterized by a lower insulin excretion than the normal subject. Nevertheless such differences do lend support to the hypothesis that the diabetic patient suffers from an insulin insufficiency.

Of greater significance than the preceding is the fact that normal as well as diabetic subjects excrete minute quantities of insulin irrespective of whether or not large amounts of exogenous insulin are administered. The fate of endogenous and exogenous insulin remains unknown but our data do warrant the assumption that insulin undergoes rapid destruction within the body.

SUMMARY AND CONCLUSIONS

1. A method is described for the assay of insulin excreted in the urine by man.
2. The daily insulin excretion was determined in normal and diabetic subjects some of whom received exogenous insulin.
3. The average daily excretion by normal subjects was 0.16 ± 0.04 units per day. The average excretion by mildly diabetic subjects who did not require insulin for the regulation of their disease was 0.07 ± 0.03 units per day. This difference was found to be statistically significant.
4. Only minute fractions of large amounts of exogenous insulin were excreted by either normal subjects or patients with diabetes mellitus.

5. Insulin-resistant patients who received large amounts of insulin appeared to excrete a smaller amount of exogenous insulin than did normal subjects also receiving large amounts of insulin.

6. The data warrant the assumption that insulin undergoes a rapid destruction within the body.

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LETTER FROM THE EDITORS

At times it is the duty of editors to call attention to practices in the writing of scientific papers which indicate either a grave lapse from literary etiquette or a cloudy concept of intellectual honesty. We refer to the practice of lifting ideas or even large portions of text from previously published articles with no hint of the source and no acknowledgment. Such plagiarism may consist of borrowing paragraphs and pages or copying a bibliography intact with errors and all. We have had papers submitted parts of which were directly copied from readily available medical journals, or the changes made were so slight as to be inconsequential. While the original author may feel flattered to have his ideas accepted so utterly, the filcher will be found out soon or late to his sorrow and the embarrassment of his editors.

Scientific data and clinical observations are not the proprietary right of those who gather and record them. They are published for such general use as may be appropriate. It is a great solace to one doing research to have his own work approved and accepted by others. This is a legitimate reward. A passion for anonymity does not haunt investigators. It is proper that recognition be given those who toil to advance the fronts of knowledge and provide a basis for wisdom. This custom has a long and honored tradition. It was practiced by Aristotle who went to great pains to give credit by name and reference to those whose work he sought to integrate and interpret.

We are not concerned with the legal aspects of patent laws or those which deal with plagiarism. We are concerned with the moral and ethical implications of a writer trying to take credit for the work of somebody else. Honesty cannot be compartmentalized and one looks askance on the data presented in such a background.

Progress in investigation is of necessity a derivative process and it has been remarked that scientists stand on the shoulders of those who have gone before. Science is a mosaic in which new pieces must be fitted against the old. Similar ideas may occur independently and simultaneously to widely separated workers whose factual data and thought processes follow a common path. We are not worried about dead heats in the races for priority. When papers are accepted for publication the proviso is usually attached that the same material is not sent to other journals. To this we would add the proviso that credit be given where credit is due and that medical writers avoid the temptation to borrow someone else's work.

THE EDITORS

PROCEEDINGS OF THE FORTIETH ANNUAL MEETING OF THE
AMERICAN SOCIETY FOR CLINICAL INVESTIGATION
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MEDICINE—ITS MENTAL CLIMATE

By J. S. L. BROWNE

Mental climate is a phrase used by J. W. N. Sullivan to describe the ambience within which grow and develop the philosophy, the science, the art of an era or time. I trust you will forgive my vagaries in tracing those trends in medicine of our era which may reveal its mental climate since I believe that clinical investigation as a part of medicine grows and develops under the influence of this mental climate. We tend to spend too much of our time in air-conditioned rooms and fail to look at the weather outside.

What is a trend? It is the way or direction in which a process or subject is changing or progressing. It is not possible for a single observation to determine the trend; our day to day observations of the facts as they are will not reveal it clearly. Only by drawing a graph can we detect in which way the process is going. It is easier to see a trend in the past than in the present or the future and this is so of medicine as of other subjects. We have spoken so far objectively, as if we stood outside and were observing the trend in medicine. We, however, form part of that process throughout our lives. We recapitulate in our medical education, in part at least, the evolution of that living being which is medicine.

Growth and development is a property of life. Plants, animals and man each possess certain attributes in common. In the plant world the tree springs from the seed, grows, develops a trunk, branches, twigs, leaves, flowers—these are fertilized—then again the seed, and this process is repeated. In the animal world the individual starts as the fertilized ovum, develops, matures, procreates, ages and dies—continuing in its progeny. Man continues to exist not only by his physical continuance in his children, but by the transmission of his emotions and thoughts. He not only procreates, he creates.

All living beings are under the influence of their heredity and environment. Man is conditioned not only by his physical environment but by being taught to call things by certain symbols or words, which change their meaning in different lands and at different times. He is influenced by the religious, scientific, philosophical and social aspects of his day and all of these are conveyed to him through words written or spoken. However much he changes, there are certain fundamental emotions, ways of thought and concepts which keep recurring in each generation—the spirit of human nature—which the great philosophers, artists, and perhaps statesmen of each era interpret for their time. One of his fundamental urges is the desire for certainty which he never attains.

Let us see how medicine as a living being exhibits the attributes considered above, remembering that medical men themselves are the expression in their time of the development of the subject and recapitulate within their growth the growth of medicine itself. A botanical allegory of medicine may be drawn: Development begins in the seed of a fundamental science. Let us take the development of morphological pathology and gross anatomy; in the last century it grew into a huge tree, it specialized into many branches, it sheltered and shaded medical men from the sun of uncertainty. It flowered and continues to flower in its application to medical practice—cross fertilized, it formed part of the development of the newer trees of physiology, biochemistry and biophysics. Those seeds which fell close under the thick shade of its leaves failed to develop or were stunted because of the very protection which it offered from the sun of uncertainty. I have used morphological pathology as an example because its great development occurred in the immediate past of medicine and its influence on medical thought is still great. The same occurs in all sciences which are applied to medicine. Bacteriology, physiology, biochemistry and biophysics are forming part of the growth and differentiation of medicine of the present day.

What are the effects of this growing process on medical education and thought? The medical man, like other men, demands certainty, for which he constantly looks to the science of medicine. He also has a certain continuous medical spirit which must be satisfied, the personal relation to the patient. He seeks to satisfy this by practicing the art of medicine. This art is the practical expression of his application of the science of medicine as he knows it guided by the spirit which is within him. The medical teachers of 40 years ago had had their training in the science of medicine almost entirely in morphological pathology and bacteriology. There had been established a correlation between certain signs and symptoms in the living patient and the appearance of organs at autopsy. These correlations had been given labels, had been placed in pigeon holes and the teacher could only be certain that he had put the right label on by examination of the patient at autopsy or possibly at surgical operation. The teacher also possessed a clinical acumen derived from observation by means of his own senses. In addition in many instances as a successful family physician he considered his patient, whom he often looked after for many years, as a person. On the other hand in teaching he sometimes overemphasized the science of medicine and

came to regard a public ward case as increasingly interesting as the inverse square of the distance of that patient from the autopsy table. The students were taught on the wards, the outdoor was regarded as a means of discovering interesting cases for the wards and the student had no opportunity to see the art of medicine guided by its spirit as practiced in the teacher's office and when he visited the home.

To the next generation of teachers brought up in this tradition there came the increasing capacity to extend the facts gained by their own senses, by means of instruments and tests revealing the physiological and biochemical aspects of the processes going on in the patient. The number of facts available in any one patient increased and is increasing immensely. The growing trees of the various medical sciences and of medicine itself (as a sort of banyan tree which includes the others) developed more and more branches and twigs of specialization. On the one hand this growth led to a greater likelihood of attaining intellectual certainty within the patient's lifetime, to a better recognition of disease as a physio-pathological process and, through the development of special therapeutic agents, to a greater capacity for prevention, amelioration or even cure of these disease processes. This increased the interest in the physio-pathology of disease, the facts attainable during life increased the interest in the patient apart from his proximity to the autopsy table and the tendency to therapeutic nihilism was overcome. On the other hand the interest in intellectual certainty led physicians in the teaching wards to the collection of more and more facts by more and more laboratory investigations. Twenty tests were regarded as equal to one autopsy for the attainment of certainty. The brilliant flowers of laboratory facts were admired without realizing that they only flowered because of their connection with the previous development of medicine and because they remained in truth connected to the history and the physical examination. Plucked from the parent stem they tended to wither. The spiritual continuum of medicine, the recognition of the person in the patient, the sap which begins in the roots, flows through the trunk, leaves and flowers of medicine and connects and gives life to the whole structure was not clearly presented to the medical student.

In this era, which extends to the present, the older clinician feels that the newer one is not a doctor because he relies too much on the laboratory. It seems to me that there is no essential difference between the facts obtained by the senses and those obtained by the extension of these senses. What remains essential is the capacity of the doctor from his experience of man and of medicine to obtain the facts regarding the abnormal processes occurring in the person, beginning with history, followed by physical examination and then, by use of his experience and acumen to observe what further extension of his senses are most useful—what laboratory tests are needed.

As mentioned before, one great difficulty in hospital practice is the knowing of the person, his emotional and social environment. This combined with the desire for certainty, as we have said, led to a tendency to the fac-

tualization and scattering of the patient all over the lot of specialism. A reaction against this is occurring among the public and, I think, among younger medical men and students. This again is reflected in the growth of new trees in medicine—psychiatry and social medicine. These subjects will provide new ways of obtaining facts about the patient and about his person, through the senses of the doctor, by history taking either by himself or by some other person. It should be remembered, however, that these facts that approach the person most closely need all the more to be integrated into the whole in the spirit of medicine, by the doctor be he specialist or family physician. The patient may be as badly factualized or scattered by these new disciplines as by the older ones and they are just as subject to the error of regarding the label or pigeon hole as the truth itself and not only a convenient arrangement of the truth.

I spoke some time ago about the importance of symbols and words to man and how the meaning of these words changes with time. The establishment of pigeon holes or labels, that is, the naming of disease entities, is done by men who observe, by the methods available to them in their day, the correlation between signs and symptoms in the patient and some reference standard either morphological at autopsy; or biochemical or physiological, which is a constant feature accompanying these signs and symptoms and regarded as being in some way casually connected with them.

There is sometimes confusion as to what constitutes a disease entity. For example the word amenorrhoea obviously describes a symptom and the nature of the physio-pathological process underlying this symptom may be varied. In amenorrhoea one may recognize a quantitative aspect, an individual may be just not menstruating and any form of therapy will be effective, or may be ovariectomized when these same methods of therapy may be ineffective. It is perhaps less generally recognized that diabetes mellitus is also the name of a symptom as it was originally labelled. The concepts as to its physio-pathological basis have varied and each succeeding generation of doctors has used this same label for different things, all of them desiring certainty and saying: only if the signs and symptoms can be fitted into the particular pigeon hole which I have established on the basis of my interpretation of the science of medicine may they be tagged with this originally purely symptomatic label. In the case of rheumatic fever, this disease entity has been under the morphological sanction of the occurrence of the Aschoff body, which means strictly that unless the patient dies rheumatic fever cannot be proven. However, prolonged clinical experience has established the probability that if the patient has a certain number of signs and symptoms the correlation is good enough to justify the label. On the other hand the capacity to say this is not rheumatic fever is more limited. There are many cases which are almost-not-quite rheumatic fever which may easily be less marked examples of the same physio-pathological process. To establish a pigeon hole and say that this fits and this does not fit is satisfying for certainty and enables the doctor to move thereafter in an

easy manner along the well worn grooves of recognized prognosis and therapy. However, if a new sanction or index of the physio-pathological process is found, capable of indicating the quantitative aspect of this process, its acceptance will be difficult because it will be said that it occurs in cases which are not rheumatic fever and the false conclusion drawn that, because the signs and symptoms do not fit exactly into the pigeon hole, they have no relation to the physio-pathological process underlying the pigeon hole or label. Let us consider the meaning of the word functional. When I was a student it referred to a type of disturbance not accompanied by detectable morphological change, or to the physiological and biochemical disturbances resulting from an organic lesion. It now is used as being equivalent to psychogenic and this in turn to inexplicable and to mean that no physiological or biochemical changes are detectable to account for the symptoms. You will see that this change does keep the meaning in the same relative position in the tree of medical growth. In the days of morphological sanctions it meant that no cause could be found; in the days when physiological and biochemical sanctions have been added it still means this. This changing use of words causes great confusion between medical men trained at different stages of the growth of medicine even in the same generation. It also leads to great difficulty in clinical investigation since the investigator's thinking is conditioned by the concepts of disease entities and of words, which he has learnt during his medical training.

The trend of medicine is not often seen by those taking part in it. The medical man himself grows, branches, puts out leaves, flowers and goes to seed—some grow and flower for a longer, some for a shorter period. He does this within the growth of medicine itself which continues in other aspects beyond him. He has learned at a particular stage of medicine, that of his undergraduate and post-graduate days, and it becomes increasingly

difficult for him really to build into his thinking concepts which develop years later.

Each generation of medical men applies in its time the science of medicine as known at the time of its training to the practice of medicine and in turn advances the growth of the subject.

There is thus the science of medicine—the methods of obtaining facts and knowledge of the meaning of those facts; the art of medicine, the application of these facts to actual practice and the spirit of medicine—that deep sense of relationship to a person, demanding sensitivity, fine perception, true sympathy and wisdom. It should be recognized that the art of medicine changes. The doctors of yesterday were not good doctors only because they used the methods available in their time. The mere use of modern methods of laboratory diagnosis does not make a doctor a good or a poor one. It is the failure to integrate the facts obtained by any method and the failure to remain conscious of the spirit of medicine which makes a poor doctor be he general practitioner, internist, surgeon or psychiatrist.

The problem of medicine today is how can the reintegration of the patient as a person be achieved in the welter of facts developed through the science of medicine, and in view of the fact that no one man can know any but a small fraction of them. Each doctor can remain conscious of the spirit of medicine, even though he be a specialist and each family physician can do his best in relation to the stage of his own growth in the art of medicine.

In these ramblings I have tried to indicate something of the mental climate in which medical men and therefore medicine have grown and developed. Part of this climate has been the same for thousands of years, part of it has only recently developed but all of it has its impact on the growing medical man and on medicine itself.

ABSTRACTS

A Prothrombin Activator in Serum, and its Significance in Certain Hemorrhagic Diseases. BENJAMIN ALEXANDER, (by invitation) ANDRE DE VRIES, and (by invitation) ROBERT GOLDSTEIN, Boston, Massachusetts.

Normal serum contains a substance which can accelerate conversion of prothrombin to thrombin by thromboplastin plus calcium. This fact, reported by Ware *et al.* and confirmed by us, is demonstrable by the enhanced prothrombin activity of plasma-serum mixtures. A study of this serum activator in certain hemorrhagic disorders revealed that:

(a) Hemophilic blood consumes abnormally small amounts of prothrombin during coagulation. Hemophilic serum is devoid of activator. Adding normal plasma to hemophilic blood *in vitro* or *in vivo* accelerates its coagulation but fails to induce normal prothrombin consumption or serum activator evolution.

(b) In idiopathic thrombocytopenic purpura, the serum is similarly rich in unconsumed prothrombin and low in activator despite normal clotting time of the shed blood.

(c) Sera from dicumarolized subjects with hypoprothrombinemia are also low in serum activator.

Evolution of activator during coagulation is accordingly closely related to prothrombin consumption. It is proposed that this prothrombin activator furthers hemostasis by accelerating conversion to thrombin of additional prothrombin in the surrounding blood. Thus, *in vitro* and *in vivo*, clotting is hastened and the clot is propagated. Failure to evolve normal amounts of activator in the above disorders may explain their hemorrhagic phenomena despite relatively normal clotting time. Low serum activator may also explain the effectiveness of dicumarol in preventing thromboembolism.

Significance of Blood Carbonic Anhydrase Activity in Anemia. M. D. ALTSCHULE and (by invitation) H. D. LEWIS, Boston, Massachusetts.

A new method for estimation of blood carbonic anhydrase activity at body temperature has made possible the more accurate evaluation of the role of this enzyme in the symptomatology of anemia. The studies show that patients with anemia due to hemorrhage, febrile states, uremia, acute hemolysis, and, in most instances, leukemia, exhibit decreases in blood carbonic anhydrase activity parallel with diminution in hemoglobin and hematocrit levels. Patients with pernicious, "refractory," and, in some instances, nutritional anemias show much higher levels of blood carbonic anhydrase activity than would be anticipated from the erythrocyte count and hemoglobin and hematocrit levels. In view of the importance of the enzyme in carbon dioxide transport, especially under conditions of acceleration of the circulation, it is concluded that the observations are significant in relation

to variations in the degree of dyspnea, commonly associated with types of anemia.

A Type Specific Protein from Pneumococcus. ROBERT AUSTRIAN (Introduced by Colin M. MacLeod), New York, N. Y.

The capsular polysaccharide has long been recognized as the principal determinant of pneumococcal type specificity. The presence also of a hitherto undescribed type specific protein has now been demonstrated. By injecting rabbits with vaccines prepared from rough variants of pneumococcus types I, II, III and VIII, antisera have been obtained which react specifically with a protein extracted from pneumococci of the homologous type. The type specific protein can be extracted from both smooth and rough variants by heating the organisms in dilute acid.

The type specific proteins of pneumococci share certain chemical properties with M proteins of Group A hemolytic streptococci, but no cross reactions have been observed among the four pneumococcal proteins studied and those of twenty-nine types of Group A streptococci. Antisera against pneumococcal type specific proteins give negligible protection against homologous encapsulated pneumococci in mice. When a rough variant of pneumococcus type II is transformed *in vitro* to capsular types I or III, it retains the specific protein of type II, demonstrating thereby the independent variability of these two type specific characters. If transformation occurs in nature, its detection should be possible by studying a sufficiently large number of pneumococcal strains.

Does One Pituitary Hormone Stimulate All Three Functions of the Adrenal Cortex? FREDERIC C. BARTTER (Introduced by Fuller Albright), Boston, Massachusetts.

The adrenal cortex produces three functional types of hormones: (1) the "Na" hormones (desoxycorticosterone-like) which promote the retention of sodium and chloride and the excretion of potassium, (2) the "S" hormones ("11-oxycorticosteroids") which maintain the blood sugar in the fasting state by means of glyconeogenesis, and (3) the "N" hormones ("17-ketosteroids") which promote anabolism of protoplasm and, in the female, control the production of axillary and pubic hair.

Before adrenocorticotrophic hormone (ACTH) became available, the evidence indicated that the "Na" hormones were produced independently, the "S" hormones through stimulation by ACTH, and the "N" hormones through stimulation by some second pituitary tropic hormone (possibly the luteinizing hormone) which is not released before puberty. Thus, the rat is capable of retaining sodium and chloride in the absence of the pituitary; the pre-

pubertal child produces adequate "S" hormones but no "N" hormones.

With the use of purified ACTH the problem has been reexamined in human subjects.

The metabolic changes following ACTH include: (1) retention of sodium and chloride, with loss of potassium; (2) loss of nitrogen, phosphorus and calcium, with increased excretion of "11-oxysteroids"; and (3) increased excretion of 17-ketosteroids.

This evidence reopens the question as to whether ACTH stimulates all functions of the adrenal cortex.

Influence of Tobacco Smoking upon the Effectiveness of Antacid Therapy and Management of the Peptic Ulcer Patient. ROBERT C. BATTERMAN and (by invitation) IRVING EHRENFELD, New York, New York.

The influence of tobacco smoking upon the management of the peptic ulcer patient was studied in 108 ambulatory patients. In terms of the ability of the patient to respond to antacid therapy, the fifty-six patients who continued to smoke presented a satisfactory clinical response in only 46.8 per cent of the trials. On the other hand, the thirty-nine patients who never smoked presented satisfactory improvement in 85 per cent of the trials. The incidences of acute exacerbations during the period of antacid therapy reveals very strikingly the influence of smoking upon the cause of the peptic ulcer syndrome. The patients who smoked presented an acute exacerbation in 34, or 53 per cent of the trials in contrast to the non-smokers who had exacerbations in 17.5 per cent of the trials. In every case of the group of 26 patients who stopped smoking of their own accord before seeking treatment, there was a satisfactory clinical response to antacid therapy. The incidence of exacerbations was 11.5 per cent. Thirteen patients resumed smoking after clinical improvement occurred. Eleven showed an immediate regression of their disease with increased symptoms of acute exacerbations.

Effects of Proteinuria on the Kidney. JAMES H. BAXTER and GEORGE C. COTZIAS (Introduced by Homer F. Swift), New York, N. Y.

To determine effects on the kidney of prolonged, continuous proteinuria, comparable groups of young rats were given, for periods up to 6 weeks, twice daily intraperitoneal injections (1 cc./10 gm. body wt.) of 3 per cent solutions of *gelatin*, *human albumin*, *bovine γ -globulin*, *casein hydrolysate*, 1 per cent *urea*; the controls received the solvent solution (0.45 per cent *NaCl*). Animals were given no additional fluids.

Gelatin and *albumin* produced increased proteinuria; renal enlargement by about 40 per cent developed within 24 hrs. Particularly, *gelatin* caused paleness of kidneys and droplets in proximal convoluted tubule cells (*cf.* Oliver); a large content of injected protein in cortical tissue was demonstrated. Fat appeared normal. *Globulin* apparently was best retained; sustained serum pro-

tein elevation and hemodilution, and the least marked proteinuria and renal enlargement resulted.

Casein hydrolysate and *urea* induced little or no enlargement; this suggested that enlargement was caused by tubular reabsorption of protein, rather than by protein metabolites.

Illness occasionally observed was not attributed to renal damage. A few dilated, protein-filled nephrons were seen. Proteinuria and renal enlargement rapidly receded after termination of protein injections, and subsequent blood urea, serum protein, and renal histological studies were normal.

These results seem compatible with the concept that the large, pale kidney of nephrosis is a secondary manifestation caused in part by proteinuria of glomerular origin. No evidence was obtained that proteinuria itself induces chronic or progressive renal damage, except possibly that due to tubular obstruction.

✓ *Temperature-Elevating Effect of a Substance Obtained from Polymorphonuclear Leucocytes.* PAUL B. BEESON, Atlanta, Georgia.

Fever occurs in many different types of disease, including such diverse entities as infections, neoplastic diseases, hemolytic crises, vascular accidents and mechanical injuries. The way in which these various processes affect temperature regulation is unknown, but the suggestion has been made that some agent, liberated from injured cells, acts on the cerebral thermoregulatory centers and disturbs their function.

The present work was done in an attempt to find in cells of the rabbit a substance which, on intravenous injection into normal rabbits, would cause a rise in body temperature. Four cell types were tested: erythrocytes, lymphocytes, large mononuclear cells (macrophages), and polymorphonuclear leucocytes. ✓ Only one of these—the polymorphonuclear leucocyte—caused fever. These cells were obtained from sterile peritoneal exudates, caused by injection of large volumes of physiologic salt solution. Aseptic technique and pyrogen-free materials were used in separating them from the fluid exudate. Elevations of 2–3° F. resulted from intravenous injection of approximately the number of cells normally present in the circulating blood. The rise begins in 10 to 15 minutes and reaches its peak within an hour.

When a suspension of polymorphonuclear leucocytes is subjected to mechanical lysis (by shaking with glass beads) and then centrifuged, the supernatant fluid is fully active in causing fever, whereas the cell residue has no effect. The fever-producing property disappears after heating to 75–80° C. for 30 minutes. The active substance does not dialyse through a cellophane membrane. Further studies on the nature of this substance are under way.

It seems possible that the liberation of material such as that present in the polymorphonuclear leucocyte of the rabbit plays a role in the pathogenesis of fever in certain diseases of man.

The Effectiveness of Bismuthoxy-p-N-Glycolylarsanilate (Win-1011) in the Treatment of Intestinal Amoebiasis.

D. A. BERBERIAN (Introduced by T. G. Klumpp), Rensselaer, New York.

Win-1011 is the bismuth salt of an arsenical, containing 15.7 per cent arsenic and 37 per cent bismuth, characterized by low oral toxicity. It was supplied by Winthrop-Stearns, Inc., for studies at the American University of Beirut, as 0.25 gm. tablets.

Sixty-eight cases of chronic and subacute intestinal amoebiasis were treated with *Win-1011* orally. Certain cases also received Chiniofon and/or bismuth subgallate; 32 control cases were treated with Chiniofon.

Endamoeba histolytica was demonstrated in stools of all patients prior to treatment. Each adult received 2 tablets (0.5 gm.) of *Win-1011* after each meal for 7 days (total 10.5 gm.); children received proportionately smaller doses. Chiniofon was given as 0.25 gm. tablets; a total of 11.25 gm. was given in 7 days. Medication was followed by periodic laboratory examinations (av. 9.4) over an average period of 108 days.

Win-1011 alone cleared without relapse 24/25 cases; Chiniofon alone cleared 5/11 cases. *Win-1011* combined or alternated with Chiniofon cleared 33/38 cases; *Win-1011* and bismuth subgallate alternately cleared 5/5 cases; Chiniofon with bismuth alternately cleared 10/21 cases. All patients were cleared temporarily. No side-effects of any kind occurred in patients receiving *Win-1011* alone; therapeutic response was prompt.

The Mechanism of Flattening of the Erythrocytes in Diseases of the Liver and Bile Ducts. LIONEL BERK (Introduced by Henry Jackson, Jr.), Boston, Mass.

Erythrocyte "flattening," shown by increased resistance to osmotic hemolysis and by calculations based on volume and diameter measurements, was demonstrated in the blood of patients with cirrhosis of the liver, and within a few days of the onset of obstructive jaundice and acute hepatitis. During recovery from the latter, flattening disappeared in about a week. Because the changes occurred so rapidly while the osmotic fragility curves remained symmetrical and no reticulocytosis was seen, the changes apparently occurred in circulating red cells.

Attempts to reproduce this phenomenon *in vitro* failed when normal red cells were incubated at 37.5° C. for as long as 48 hours in plasma from patients with flattened erythrocytes. However, flattening of normal red cells transfused into such patients was apparent when the "hump" on the abnormal fragility curve initially produced by their presence showed progressive diminution within 1 to 2 days and disappeared within 3 to 5 days thereafter, despite the demonstrated persistence of the normal transfused erythrocytes in the circulation.

Since the flat red cells usually showed no change in volume, an increase in the area of the cell envelope must exist. The failure to reproduce flattening *in vitro* suggests that the tissues contribute factors responsible for this change. No correlation could be established be-

tween increased osmotic resistance and bilirubin, cholesterol or bile salts retained in the plasma.

The Renal Tubular Secretion of Potassium. ROBERT W. BERLINER and THOMAS J. KENNEDY, JR. (Introduced by A. B. Gutman), New York, N. Y.

The potassium excreted in the urine ordinarily is considerably less than the amount contained in the glomerular filtrate, indicating a tubular mechanism for potassium reabsorption. We have obtained evidence that a tubular secretory mechanism for potassium is also present in the normal dog. This was first suggested by the constancy of potassium excretion after the administration of salyrgan. Following the injection of salyrgan, potassium excretion may either increase or decrease but rapidly attains a fairly constant level at which it remains despite appreciable variations in filtered load associated with changes in filtration rate (creatinine clearance). This finding could be explained by the complete reabsorption of potassium in the proximal tubule and the secretion of potassium at a constant rate in the distal tubule.

More direct evidence of tubular secretion was obtained by the intravenous infusion of potassium in dogs whose tolerance had been increased by oral administration of potassium. It has been possible to attain potassium excretion rates 45 per cent greater than the simultaneous glomerular filtration of potassium (plasma potassium uncorrected for Donnan equilibrium multiplied by creatinine or inulin clearance).

Experiments in progress indicate that a similar mechanism for tubular secretion of potassium exists in man.

Catheterization of the Coronary Veins and the Measurement of Coronary Blood Flow in Man. R. J. BING, W. T. GOODALE, J. E. ECKENHOFF, J. C. HANDELSMAN, J. A. CAMPBELL, H. E. GRISWOLD, L. D. VANDAM, M. HARMEL, J. H. HAFKENSCHIEL, M. LUBIN, and S. S. KETY (Introduced by Alfred Blalock), Baltimore, Maryland.

The coronary blood flow of the dog has been determined using the nitrous oxide method of Kety and Schmidt. This report deals with the catheterization of the coronary sinus and the middle cardiac vein and with the determination of the coronary blood flow in man.

The procedures were carried out largely on patients with congenital heart disease and on a small group of individuals with peripheral vascular disorders without cardiac involvement. A special catheter designed by one of us (W. G.) was used in the majority of cases, since sampling of coronary blood through the standard Courmand catheter was difficult. Position of the catheter in the coronary veins was established by fluoroscopic visualization, blood oxygen contents, and by pressure measurements.

Coronary veins were intubated in nineteen individuals. The arteriovenous oxygen differences ranged from 8 to 18 vol. per cent. In five patients the coronary blood flow could be measured. In two of these the flow was 60 and 80 cc./min./100 gms. of heart tissue respectively.

tient with osteomalacia and the Fanconi syndrome had decreased C_{Inulin} , and C_{PAH} with a high filtration fraction.

Renal tubular bicarbonate reabsorption was measured once in each syndrome. Starting at acidotic levels heavy loads of sodium intravenously resulted in increased urine alkalinity, and bicarbonate excretion up to 0.4 milliequivalent per 100 cubic centimeters of glomerular filtrate while plasma bicarbonate was well under 25 milliequivalents per liter. In agreement with Pitts observations, two normal subjects began bicarbonate excretion only after plasma bicarbonate exceeded this level. Simultaneous bicarbonate and sodium excretion, measured only in the Fanconi syndrome, showed that most of the sodium being wasted was in combination with bicarbonate.

The clearance measurements suggest generalized renal impairment, but greater relative tubular than glomerular dysfunction in both syndromes. In each group the acidosis could be partially explained by the inability of the kidneys completely to reabsorb all bicarbonate filtered at low plasma levels of this anion.

The Oxygen Consumption of the Human Kidney.

WALTER H. CARGILL and JOHN B. HICKAM (Introduced by W. M. Nicholson), Durham, North Carolina.

Samples of renal venous blood were obtained by the catheterization technique and compared with simultaneous samples from the femoral artery for oxygen and sodium p-aminohippurate (PAH) content. Intravenous infusions of inulin and PAH were given and urine collected according to the usual clearance methods. Renal blood flow was calculated from the rate of PAH excretion, the arterial-renal venous PAH difference, and the hematocrit reading. The oxygen consumption of the kidney was estimated from the arterial-renal venous oxygen difference (renal oxygen extraction) and the renal blood flow. The glomerular filtration rate was measured at the same time by the inulin clearance. All subjects were studied under basal conditions.

A total of 35 subjects have been studied. For the sake of comparison, these have been divided into three groups: (1) ten patients without hypertension or clinically apparent renal disease, (2) seventeen patients with chronic pyelonephritis or essential hypertension, and (3) eight patients with subacute glomerulonephritis. In the normal subjects the mean renal oxygen extraction was found to be 1.4 vols. per cent, and the mean renal oxygen consumption 16.0 cc./min., standard deviation 2.8. The patients with chronic nephritis and hypertension demonstrated a normal oxygen extraction (1.5 vols. per cent) but a moderate to marked reduction in renal blood flow, so that the renal oxygen consumption for the group as a whole was below normal limits (mean 9.0 cc./min.), the decrease in oxygen consumption for each individual being a function of the decrease in blood flow. In this group of subjects a direct relation between renal blood flow and oxygen consumption was apparent. In contrast, the patients with subacute glomerulonephritis showed a reduction in oxygen consumption (mean 7.0 cc./min.) due almost entirely to a decreased oxygen extraction (mean 0.7

vol. per cent), since the renal blood flow of most of these patients was within normal limits.

In the entire group of 35 patients studied a positive correlation between glomerular filtration rate and renal oxygen consumption was apparent. No correlation was evident between the oxygen consumption and the degree of tubular reabsorption of water as measured by the inulin U/P ratio.

It is hoped that these findings will constitute a physiological basis for the clinical differentiation of patients with kidney disease into two groups: (1) those with a disturbance of renal metabolism secondary to occlusive vascular disease (chronic pyelonephritis and essential hypertension), and (2) those with an alteration in renal metabolism with a normal renal blood flow (subacute glomerulonephritis). Whether the decreased renal oxygen consumption in the second group is due to impaired glomerular filtration or primary tubular dysfunction is yet to be determined.

The Use of Radioactive Phosphorus in Measuring Plasma Phospholipide Formation in Patients with Cirrhosis of the Liver. The effects of Treatment with Methionine. DAVID CAYER and W. EUGENE CORNATZER (Introduced by David T. Smith), Winston-Salem, N. C.

In animals on deficient diets choline and methionine prevent fatty infiltration of the liver. They also stimulate the formation of liver phospholipides which are the main source of plasma phospholipides. Clinically, methionine is useful in the treatment of human beings with chronic hepatitis—an early stage of cirrhosis—as well as those with more advanced disease and ascites. To determine if the benefit is due to an effect on phospholipide formation, hospitalized patients with cirrhosis and normal individuals were given intramuscular injections of radioactive phosphate. At various time intervals after injection, radioactivity and total phosphorus content were determined in the lipid and in the inorganic fractions of plasma. On the basis of the specific activity—time curves obtained on the phospholipides of normal individuals, the 24 hour level was selected for comparison with that found in patients with cirrhosis. The specific activities (even when adjusted in relation to the specific activity of the inorganic phosphate in plasma) show considerable variations both in the control group and in patients with cirrhosis. No significant difference was found between the two groups. The possible effect of methionine on phospholipide turnover was investigated by reinjecting radiophosphorus after 30 days of treatment with methionine (3 gm. per day).

The Incidence, Character and Course of Liver Disease in Chronic Alcoholics as Determined by Needle Biopsy. THOMAS C. CHALMERS, T. LYNCH MURPHY and EDGAR B. TAFT (Introduced by Clark W. Heath), Cambridge, Massachusetts.

Liver biopsies were obtained within a few days of admission to the hospital from 24 patients manifesting incipient or active delirium tremens. Physical and lab-

oratory signs of liver disease were minimal or absent, but histologically all of the 24 livers were abnormal. In seven biopsied a second time there was improvement after treatment with a regular hospital diet.

These patients could be classified according to the Bowman-Jellinek scheme as either steady or intermittent drinkers. The two groups were equal in number and similar in respect to age and duration of alcoholism. The steady drinkers ate at least one good meal a day, were regularly employed and usually entered the hospital because of an infection. The intermittent drinkers neither ate nor worked while drinking and usually entered the hospital with uncomplicated delirium tremens. As shown in the table, the extent and character of the liver injury seemed to depend on the alcoholic habits of the patient. It is concluded that the liver is abnormal in chronic alcoholics after a bout severe enough to terminate in delirium tremens and that the steady drinker is more likely to show the histological picture of alcoholic cirrhosis.

	Intermittent drinkers	Steady drinkers
Total	12.	12
Fatty metamorphosis	12	4
Fatty infiltration	0	8
Fibrosis—definite	3	7
Fibrosis—slight	1	3
Necrosis	1	6
"Alcoholic" hyaline	0	7
Unidentified pigment—moderate or more	8	1

The Effect on Respirations and Blood Pressure of Electrical Stimulation of the Orbital Surface of the Frontal Lobe and of Frontal Lobotomy in Man. WILLIAM P. CHAPMAN, (by invitation) ROBERT B. LIVINGSTON, and (by invitation) KENNETH E. LIVINGSTON, Boston, Massachusetts.

During light pentothal anesthesia transcortical electrical stimulation of the orbital surface of the frontal lobe produced arrest of respirations in the expiratory phase in six out of eight patients and elevation of blood pressure in five out of eight patients. Respiratory and blood pressure responses had consistent latency and recovery periods and were obtained independently or together depending on the area stimulated. The magnitude of blood pressure elevation was from ten to twenty mm. of mercury, these changes being from two to four times as great as the maximal variation during the control period. Blood pressure was recorded by a Hamilton manometer and respirations by a pneumatic cuff.

A significant lowering of elevated blood pressure in eleven mental patients, extensively studied, was maintained not longer than five months after frontal lobotomy.

These studies establish for the first time in man that stimulation of the orbital surface of the frontal lobe arrests respirations and elevates blood pressure. Frontal lobotomy apparently does not result in permanent lowering of blood pressure. The nature of this mechanism, the anatomical pathways involved, and the importance of

these observations relative to the role of emotions in modifying respirations and blood pressure are not understood.

The Electrolyte Content of Thermal Sweat as an Index of Adrenal Cortical Function. JEROME W. CONN and (by invitation) LAWRENCE H. LOUIS, MARGARET W. JOHNSTON, and (by invitation) BETTY J. JOHNSON, Ann Arbor, Michigan.

Several years ago we reported metabolic evidence indicating that the process by which normal men acclimatize to heat is associated with a sharp increase in adrenal cortical activity. In the course of this study it became clear that the electrolyte composition of the sweat was reflecting changes in adrenal cortical function.

The present study indicates (1) a consistent relationship between the electrolyte composition of sweat and the degree of activity of those corticosteroids which effect salt and water metabolism (S W corticosteroids), (2) that reabsorption of electrolytes by the tubules of the sweat glands and kidneys is affected in a similar way by the action of S W hormones but that the sweat does not "rebound" toward normal under continued activity of these steroids as does the urine, (3) that A C T H is capable of stimulating simultaneously increased production and release of all three types of corticosteroids (N, S, and S W), and (4) that in various clinical states involving increased or decreased function of the adrenal cortices, the electrolyte composition of the sweat affords clear evidence of the disturbance.

Results.

(1) *D C A* in normal humans produces a sharp fall in the concentrations of both Na and Cl of sweat and a rise in the concentration of K. Increased reabsorption of Na is more intense than that for Cl. This effect persists for as long as *D C A* is given and does not "rebound" on continued administration of *D C A*. Upon cessation of *D C A* an intense rebound occurs.

(2) *A C T H* in normal humans produces precisely the same effects upon the electrolyte concentrations of sweat and urine as those produced by *D C A*. Under continued administration of 120 mg. per day for 8 days, the rebound of the electrolyte concentration of urine had occurred by the third or fourth day of injection while that of the sweat did not rebound until two to three days after the last injection (the 17-ketosteroids had already returned to baseline).

In parallel but different in time relations, there occurred negative nitrogen balance, great loss of carbohydrate tolerance, overall retention of Na and Cl, and loss of K, typical hematological changes, initial increase in uric acid excretion, and a four to fivefold increase 17-ketosteroid excretion. No change was observed in the gamma globulin fraction of the serum proteins by electrophoresis nor in immune titers.

(3) *Sweat tests in patients with adrenal cortical dysfunction (14 cases).*

(a) Normal range of Cl conc. in m.eq. per liter	17.5 to	58.0
(b) Adrenal cortical carcinoma with Cushing's syndrome	1.7 and	2.7
(c) Adrenal cortical carcinoma with adrenogenital syndrome	5.7 and	9.1
(d) Cushing's syndrome without carcinoma (2 cases)	5.9 to	13.5
(e) Pan-hypopituitarism (3 cases)	68.0 to	75.0
(f) Addison's disease (7 cases)		
Untreated	105.0 to	122.0
Treated	25.0 to	63.0

Na concentrations show similar degrees of difference between normals and patients. K changes are smaller in degree but significant.

Improvement in Pulmonary Function after Anticholinergic Agents in Spontaneous and Methacholine-induced Asthma. JOHN J. CURRY, JOB E. FUCHS and SAMUEL E. LEARD (Introduced by William L. Fleming), Boston, Massachusetts.

Sporadic attention has been focused on the role of the parasympathetic nervous system in bronchial asthma. We have confirmed the abnormal responsiveness of the tracheobronchial tree to cholinergic substances using agents such as pilocarpine, neostigmine, furfuryl trimethylammonium iodide, methacholine and acetylcholine itself. The order of this surprising sensitivity and the resemblance of induced attacks to spontaneous asthma suggested that a survey of anticholinergic agents might furnish information useful in the treatment of bronchial asthma. The method of study has been outlined in part by previous communications. In brief it involves a sharp reduction in pulmonary function tests, induced by either spontaneous asthma or the injection of methacholine. In the former cases the improvement in pulmonary function following the administration of anticholinergic agents was recorded, whereas in the latter the degree of protection afforded by them against subsequent repeated doses of methacholine was measured. In the majority of tests a 9 liter Benedict-Roth type metabolism apparatus was used.

Results indicate that contrary to popular opinion the belladonna alkaloids, atropine, 1-hyoscyamine, hyoscyne and Bellafoline afforded notable relief in spontaneous asthma and were very effective in protecting against methacholine-induced asthma.

Synthetic anticholinergic agents such as Trasentin and Syntropan with the exception of merperidine hydrochloride were not very effective. Sympathomimetic amines, such as ephedrine and Orthoxine, and also aminophylline were usually effective. Benadryl varied from no effect to slight effectiveness.

Cancellation of Fluoride Antiglycolytic Activity by Calcium and Magnesium Ions. T. S. DANOWSKI, Pittsburgh, Pennsylvania.

Release of glycolysis from fluoride inhibition by calcium and magnesium salts was studied in defibrinated

blood. CaCl_2 and MgCl_2 (20 to 80 milliequivalents per liter) in blood which contained 20 milliequivalents per liter of NaF completely or almost completely abolished the antiglycolytic effect of fluoride. In the lower concentrations, CaCl_2 proved more effective than MgCl_2 ; the reverse was true at 80 milliequivalent per liter levels. This release is related to calcium and magnesium ions, since other chlorides (KCl , NaCl , NH_4Cl) were ineffective. Furthermore, known accelerators of glycolysis in blood ($\text{PO}_4^{=}$, $\text{SO}_4^{=}$, and HCO_3^-) failed to cancel the fluoride effect. However, it was possible to show in fluoride-treated blood the characteristic acceleration which follows added $\text{SO}_4^{=}$, by introducing magnesium in excess of the fluoride present.

It seems probable that the cancellation of fluoride activity by Mg^{++} and Ca^{++} is related to the high insolubility of the fluoride salts of these cations, and possibly to the replacement of deficits of Mg^{++} and Ca^{++} . The former is supported by the comparable efficacy in this respect of Ba^{++} which also forms an insoluble fluoride, and by the failure of CaF_2 and MgF_2 to suppress glycolysis in blood.

A Study of the Physiologic Function and Histopathology of Thyroid Adenomas Using Radioactive Iodine and Radioautography. BROWN M. DOBYNS and BEATRICE LENNON (Introduced by J. H. Means), Boston, Massachusetts.

The function of thyroid adenomas was demonstrated by radioautographs made from histologic preparations of nodules removed from 90 patients who had received radioactive iodine preoperatively. Localization of function was correlated with the histologic features of the tissue including cellular pattern, cell height, colloid formation, and nuclear and cytoplasmic details. These were compared with clinical aspects.

The degree of function of the adenomas runs parallel to the degree of cellular differentiation. Single or multiple hyperfunctioning adenomas, with a distinct histologic pattern, occurred in patients having thyrotoxicosis; however, appreciable numbers were found associated with normal or subnormal basal metabolic rates. In the latter group the total hyperfunctioning cell mass was insufficient to cause elevation in basal metabolic rates (these were thought to be related to some borderline clinical features of hyperthyroidism). A depression of the cell height of the uninvolved tissue was observed in instances of hyperthyroidism arising from hyperfunctioning adenomas. Here the uninvolved tissue was practically functionless. Some non-functioning hyperplastic adenomas presented a picture of irregular but increased cell height in contrast to a uniform increase in cell height found in hyperfunctioning adenomas. From histologic aspects the non-functioning hyperplastic adenomas graded into the papillary forms of malignancy without function.

The Paradoxically Retarded Bactericidal Activity of Penicillin at High Concentrations in Vitro and in Vivo.
HARRY EAGLE, Bethesda, Maryland.

The bactericidal activity of penicillin against a particular organism may be defined in terms of three concentrations: (1) that which serves only to reduce the rate of multiplication, (2) that which causes a slow bactericidal action, and (3) a somewhat higher concentration at which the organisms are killed at a maximal rate.

With some organisms this maximal rate is unaffected by even a 20,000-fold further increase in concentration of the drug. With other organisms, however (*e.g.*, all of 4 strains of Lancefield group B β -hemolytic streptococci, 2 of 4 strains of group G streptococci, 5 of 7 strains of *Streptococci fecalis*, 2 of 4 strains of other α -hemolytic organisms, and 4 of 9 strains of *Staphylococcus aureus* and *albus*), when the concentration of penicillin was increased beyond the maximally effective level, there was a progressive and in some instances striking decrease in the rate of bactericidal action.

A similar phenomenon was sometimes observed *in vivo*. In such cases the high concentrations of penicillin afforded by the frequent administration of large doses were significantly less effective than the lower concentrations afforded by smaller doses. In the treatment of infections with these zone-sensitive organisms, the most effective method of treatment may well be repeated small doses, or a continuous infusion at a rate designed to maintain the optimally effective concentration at the focus of infection.

Evidence for the Concept that Total Lung Rest is Provided by the Equalizing Pressure Chamber. CHESMORE EASTLAKE, JR. and JOHN E. GARY (Introduced by Alvan L. Barach), New York, N. Y.

Patients with pulmonary tuberculosis exposed to an alternating pressure of 55 mm. Hg 25 times a minute reveal absence of all discernible chest motion when the resistance in the respiratory passageway is counterbalanced by a differential pressure of 4 to 8 mm. Hg. X-ray studies have been made with superimposed lead crosses that demonstrate the degree of chest motion during normal breathing and the absence of motion during exposure to equalizing alternating pressure. A motion picture (4 minutes) reveals, through animation, the variations in air density and chest pressures, which explain the mechanism of the chamber and, by means of photography of patients, the degree of total lung rest obtained.

The Pulmonary Blood Volume by a Dye Injection Method and its Relation to Pulmonary Hypertension in Certain Cardiac Lesions. RICHARD V. EBERT and (by invitation) CRAIG BORDEN, (by invitation) HERBERT S. WELLS and (by invitation) RUSSELL H. WILSON, Minneapolis, Minnesota.

In 10 normal subjects and a group of patients with heart disease, the pulmonary artery was catheterized. Evans Blue Dye was injected rapidly into the pulmonary

artery and multiple blood samples were collected from the femoral artery. The serum concentration of dye plotted against time gives a curve which, in normal individuals, is symmetrical and reaches or approaches zero before recirculation begins. The mean circulation time (CTm) is read from the curve at the mid point of its area. The volume of blood in pulmonary vessels, left heart and larger systemic arteries (PBV) is equal to the cardiac output (L/Min.) $\times \frac{\text{CTm (Sec.)}}{60}$.

In patients with mitral stenosis and exertional dyspnea of long standing but without hepatomegaly or peripheral edema, the pulmonary arterial pressure is markedly increased but the PBV is normal. In left ventricular failure due to hypertension or aortic valve lesions, the PBV is considerably increased but the pulmonary arterial pressure is less strikingly elevated than in mitral stenosis. These findings suggest that the increased pressure in mitral stenosis is not due entirely to increased pressure in the left auricle but is due in part to pathological changes in the pulmonary vessels.

Improvement of Active Liver Cirrhosis in Patients Maintained with Amino Acids Intravenously as the Source of Protein and Lipotropic Substances. (By invitation) R. D. ECKHARDT, (by invitation) W. W. FALLOON, and C. S. DAVIDSON, Boston, Massachusetts.

Three patients with active cirrhosis of the liver were treated for 11, 18, and 20 days with a purified diet devoid of protein. The diet contained no source of the vitamin B-complex except choline in small amounts (30 to 100 mgm. daily). A capsule containing vitamins A, C, D, B₁, B₂, niacin, B₆, and pantothenic acid was given daily.

Protein was supplied intravenously as an amino acid mixture (Merck) prepared by the acid hydrolysis of casein, devoid of peptides, glutamic and aspartic acids, and supplemented with dl-tryptophane and glycine. The days' protein (50 to 100 Gm. of amino acids containing from 2.0 to 4.2 Gm. of methionine) was administered in one rapid intravenous injection each morning.

All three patients maintained a positive nitrogen balance with an average retention of 3 Gm. of nitrogen daily in spite of a loss in the urine of from 3 to 14 per cent of the amino nitrogen administered. The urinary excretion pattern of the "10 essential" amino acids was similar to that for normals after the same infusion (microbiological assay).

The amino acid mixture was well tolerated clinically. Hyperaminoacidemia and azotemia were not observed, nor was there a progressive increase in the urinary excretion of amino nitrogen. Improvement in liver disease was observed in all three by a progressive decline in the serum bilirubin and by improvement in clinical condition. A slight weight gain occurred.

It is concluded that not only are intravenous amino acids well tolerated by patients with active liver disease, but also that clinical improvement may occur when amino acids are the sole source both of nitrogen and of lipotropic substances except for small amounts of choline.

A Study of the Changes in Plasma Volume, Renal Function, and Water and Salt Balance Induced by Repeated Administration of Human Plasma Albumin to Patients with the Nephrotic Syndrome. HOWARD A. EDER, FRANCIS P. CHINARD, ROGER L. GREIF, GEORGE C. COTZIAS, ALMA HILLER, D. D. VAN SLYKE, and HENRY D. LAUSON (Introduced by Oswald T. Avery), New York, N. Y.

Salt-poor human plasma albumin was administered to patients with the nephrotic syndrome to obtain quantitative information regarding the mechanism of albumin-induced diuresis.

Albumin was administered daily for 1 to 3 months in doses ranging from 0.5 to 1.5 grams per kilogram. One month control periods preceded and followed albumin administration. At weekly intervals changes in the clearances of endogenous creatinine, urea, chloride, and protein were measured continuously during a 24 hour period in which each voided urine comprised a clearance period. On these days plasma volume and protein concentration were determined at frequent intervals. Para-aminohippurate clearances were measured every 2 to 4 weeks. Day to day changes were followed by determination of the total 24 hour clearance of endogenous creatinine and the excretion of urea, chloride, and protein.

Administration of albumin was followed by acute increases in plasma albumin concentration, plasma volume, and total circulating albumin. The renal blood flow and glomerular filtration rate increased during this same period; concomitantly the excretion of water, chloride, and protein increased.

The data may help to clarify the relationship of water and salt excretion to the processes of glomerular filtration and tubular reabsorption, which are ultimately responsible for the accumulation and elimination of edema fluid.

Studies on the Role of the Adrenal Cortex in Protein Metabolism. FRANK L. ENGEL and (by invitation) SARA SCHILLER, (by invitation) E. I. PENTZ, and (by invitation) PHILIP K. BONDY, Durham, N. C.

Based on the relatively constant accumulation rate of urea N following nephrectomy in the rat, a method has been devised capable of detecting changes in nitrogen metabolism of the order of 1.0 mg. N/100 gms. body weight for periods as brief as one hour. The relation of adrenal cortical extract (A.C.E.) to protein metabolism was studied by this technique with the following results: (a) An increase in protein catabolism begins two to three hours after A.C.E., characterized by approximately equal increments in urea N each hour for the next three hours; (b) An intravenous injection of an amino acid mixture (Merck's VuJ) results in a prompt increase in urea during the subsequent hour, less in the second hour, and none in the third; (c) A.C.E. plus VuJ yields no more urea N than either alone, 70 per cent of the urea appearing in the first hour, i.e., the response is as if VuJ alone were given. Liver glycogen levels are significantly increased after VuJ and VuJ plus

A.C.E.; (d) Intravenous glucose two hours after A.C.E. inhibits the usual increase in urea N but does not prevent that after VuJ. Liver glycogen values are increased after glucose and glucose plus A.C.E.; (e) The rate of urea formation from injected amino acids is identical in control and adrenalectomized rats after nephrectomy. Since the increase in nitrogen metabolism after A.C.E. can be inhibited by amino acids (VuJ) or glucose, while that after VuJ cannot be prevented by glucose, and since deamination and urea formation are unaffected by adrenalectomy, it is suggested that the changes in protein metabolism after A.C.E. may not be a primary effect of the hormone, but may be secondary to the alterations in carbohydrate metabolism.

A Study of the Circulation in Pulmonary Vascular Disease. EUGENE C. EPPINGER, (by invitation) JAMES W. DOW, (by invitation) JAMES L. WHITTENBERGER, (by invitation) HENRY BREAN, Boston, Massachusetts.

Measurements of the circulation by the venous catheter technique were made in four patients with pulmonary vascular diseases of varying etiology. Each complained of dyspnea on slight exertion despite a normal vital capacity; three of "dizzy spells," and two of these three of syncopal episodes.

Pulmonary artery pressures, pulmonary capillary pressures, and cardiac outputs were measured at rest; in two patients pulmonary artery pressures and cardiac outputs were measured during exercise.

The findings at rest were as follows:

1. Elevated pulmonary artery pressures.
2. Normal pulmonary capillary pressures.
3. Normal cardiac outputs.
4. Widened arterio-venous oxygen difference.
5. Arterial oxygen unsaturation.
6. Moderate elevation of the ventilatory volume.

Two patients exhibited the following responses to mild exercise:

1. Further rise in pulmonary artery pressures.
2. Marked widening of the arterio-venous oxygen difference, in direct proportion to increased oxygen consumption.
3. Unchanged cardiac output.
4. Increased arterial oxygen unsaturation.
5. Markedly increased ventilatory volume.

It is concluded that these patients have a high resistance to blood flow, localized in the region of the pulmonary arterioles, and that their disabilities arise, in part, from the inability to increase the cardiac output to meet the demands of exercise.

The Jarisch-Herxheimer-Reaction in Early Syphilis Treated with Crystalline Penicillin. G. THOMAS W. FARMER (Introduced by J. E. Moore), Baltimore, Maryland.

1. Febrile Herxheimer reactions were observed in 41 per cent of 939 patients with early infectious syphilis

treated with crystalline penicillin G. These reactions occurred with equal frequency and severity in seronegative primary, seropositive primary and secondary syphilis.

2. In a group of 56 patients with relapsing secondary syphilis treated with penicillin, the incidence of fever was 50 per cent with the first treatment course and 38 per cent with the second course of therapy.

3. Within a wide range of penicillin dosage (10 to 120,000 u./kg.) the incidence of febrile reactions remained relatively constant (40 to 56 per cent). With extremely small doses of penicillin (1 to 5 u./kg.) febrile reactions were not observed.

4. The temporal pattern of the febrile reaction was quite uniform, and it was independent of the dosage over a wide range.

5. Febrile reactions occurred with single doses of penicillin as little as one-tenth of the amount required to render early syphilitic lesions dark-field negative.

6. With repeated small doses of penicillin (10 to 20 u./kg.) two febrile reactions were produced in the same patient. This double Herxheimer reaction was not observed after repeated large doses of penicillin.

7. The available evidence does not justify the hypothesis that the reaction is due solely to the sudden destruction of large numbers of spirochetes with the liberation of split proteins or endotoxins.

In Vitro Lysis of Leucocytes by Tuberculin and by the Serum of Patients Receiving Adrenocorticotrophic Hormone. C. B. FAVOUR and PAUL FREMONT-SMITH (Introduced by Kendall Emerson, Jr.), Boston, Massachusetts.

The tuberculin type of delayed bacterial hypersensitivity can be passively transferred with white cells from properly sensitized donors. A portion of cells from such donors when exposed to tuberculin (PPD) *in vitro* are lysed within 60 minutes. This phenomenon depends upon the presence of complement and is disease specific, as is the delayed type of skin reaction itself. On the other hand, serum from normal subjects receiving adrenocorticotrophic hormone (ACTH) or serum from an untreated patient with Cushing's disease will lyse a fraction of the leucocytes from normal donors *in vitro* in the absence of complement.

Pneumonitis of Unknown Etiology in a Group of Men Exposed to Pigeon Excreta. (By invitation) HARRY A. FELDMAN and ALBERT B. SABIN, Cincinnati, Ohio.

Twelve men, exposed in various degrees to moistened pigeon excreta in an abandoned water tower in Cincinnati, all developed illnesses of varying severity within 5 to 14 days. Three were only slightly ill with fever for 2 to 5 days while 9 were more severely affected with generalized malaise, headache, chills, non-productive cough, and marked weakness, and were febrile for 8 to 24 days. Although no significant signs were elicited on

physical examination, all of the 12 men exhibited extensive, diffuse, miliary-like infiltrates in both lung fields, giving rise to the most striking feature of this disease, the "snow-storm" roentgenogram. These x-ray changes persisted with varying diminution of intensity for 2 months or longer, but by the end of 5 to 6 months the lungs were almost clear in all but 2 patients, and there was no evidence of calcification. All recovered from the illness, but an opportunity to obtain some idea of the pathological changes of this disease presented itself when one of the men met sudden death 5 months later as a result of an acute myocardial infarction in an arteriosclerotic heart. Although the x-ray of his chest was practically clear 2 months prior to his death, multiple microscopic lesions 1 to 2 m. in diameter were found in the lungs and peribronchial lymph nodes. The lesions were in various stages of organization but in both the lungs and the lymph nodes there were many with central caseous necrosis and "Langhans-type" giant cells *unassociated* with acid-fast bacilli. Inoculation of mice with blood obtained from 11 of the 12 men during the acute stage yielded no infectious agent and serological studies as well as skin tests during convalescence indicated that the etiological agent of this disease was not that of ornithosis, Q fever, coccidioidomycosis, blastomycosis, histoplasmosis or toxoplasmosis.

Red Cell Destruction. CLEMENT A. FINCH, EDWARD D. THOMAS, ROBERT J. WALSH and REX G. FLUHARTY (Introduced by George W. Thorn), Boston, Mass.

Morphological studies have implicated the spleen, liver, and kidneys in blood breakdown. Employing erythrocytes labeled with radioiron and hemoglobin prepared from such cells, it is possible to localize accurately pigment cleared from the blood.

Labeled hemoglobin is rapidly and almost exclusively taken up by renal tissue even when given in amounts below "renal threshold." In contrast, the kidneys of rats transfused with non-viable labeled erythrocytes show only a small amount of radioactivity, while a much greater amount is found in the reticuloendothelial tissue.

For these animals, the two types of blood breakdown are easily distinguished by this technique. With *intravascular hemolysis* hemoglobin, regardless of the plasma level, accumulates in the kidney. Thereafter the radioiron can be traced to storage depots, and eventually appears in circulating red cells. This indicates that free hemoglobin is processed chiefly by the renal tissue and supports the hypothesis that hemoglobin is normally filtered through the glomerulus and re-absorbed by the tubules. In contrast, in *extravascular destruction* of red cells, the reticuloendothelial tissue takes up the damaged erythrocytes. The spleen shows a much greater capacity than the liver to dispose of red cells, as evidenced by the higher unit radioactivity found in the former organ. That this represents cell phagocytosis is indicated by the inability of reticuloendothelial tissue to handle free hemoglobin.

Clinical Experience with the Use of the ACTH Test for Adrenal Cortical Function. (By invitation) PETER H. FORSHAM, GEORGE W. THORN, and (by invitation) LILLIAN RECENT and (by invitation) A. GORMAN HILLS, Boston, Massachusetts.

Validity of the four-hour pituitary adrenocorticotropin (ACTH) response as a simple diagnostic test for Addison's disease has been confirmed in thirty-four classical cases. The mean depression of circulating eosinophils following ACTH administration was 6 per cent (-44 to $+26$) in these cases, 77 per cent (-63 to -97) in ten normals, and 73 per cent (-52 to -98) in forty non-Addisonians. Although mean fasting eosinophil levels in Addisonians (273 per cu. mm.) exceeded the normals (167) and non-Addisonians (181), there was a large overlap.

A mean increase of 21 per cent (0 to 81) in urinary uric acid-creatinine ratio was noted following ACTH administration in the Addisonians, 91 per cent (62 to 130) in the normal group and 89 per cent (28 to 172) in the non-Addisonian group. The fasting uric acid-creatinine ratio was not significantly different in the three groups (0.5).

A patient with Cushing's disease showed a low initial eosinophil count (4) and a high fasting uric acid-creatinine ratio (1.0).

Cases of mild adrenal insufficiency will respond to a forty-eight hour hormone administration in contrast to severe Addisonians. Absence of a response to intravenous epinephrine suggests hypoadrenalism associated with hypopituitarism.

The Disappearance of Edema Through Diuresis Following Artificial Elevation of Plasma Sodium and Bicarbonate. CHARLES L. FOX, JR., D. J. McCUNE, A. H. BLAKEMORE, R. GILDER and R. MOLOSHOK (Introduced by A. R. Dochez), New York, N. Y.

Edema, ascites and oliguria are usually associated with decreased plasma sodium and acidosis (Atchley, J.C.I., 1930, 9, 265). Extracellular water then migrates into cells (Peters, Phys. Rev., 1944, 24, 491; Gamble, Extracellular Fluid, Boston, 1942), resulting in reduction in plasma volume (Darrow, J.C.I., 1935, 19, 419; Winkler, J.C.I., 1944, 23, 111). Impaired excretion of water follows (McCance, Proc. Roy Soc., B, 1935-6, 119, 245). These abnormalities prevailed in one patient during anuria after diabetic acidosis; in one example of Chiari's syndrome after operation to produce porto-caval shunt followed by numerous taps of the peritoneum and pleura; and in 14 patients with the nephrotic syndrome.

Correction of low plasma sodium and bicarbonate might be hoped to augment plasma volume and subsequently increase output of urine and chloride, thereby removing anasarca. Accordingly, sodium lactate and subsequently sodium and potassium acetate were administered orally. Initially body weight increased. Plasma sodiums rose from 120-135 m.Eq. per liter to above normal; plasma bicarbonates from 9-20 m.Eq. per liter to above normal. Plasma volumes estimated from falling hematocrit ex-

panded as much as 40 per cent. Daily clearances of sodium, bicarbonate and endogenous creatinine increased several fold. Daily urine flow rose from 0.05 to over 4.0 cc. per minute as urine chloride concentration increased from 0.1 to 1.5 times the plasma value. Anasarca then disappeared.

In the balance studies the recovery of sodium approximated that anticipated from the volume of edema fluid eliminated but the chloride jettisoned was in marked excess.

Sprue—Observations on the Proteolytic Effect of Neutralized Gastric Juice on Protein Substrates, with Reference to the Activity of the Intrinsic Factor. HERBERT J. FOX (Introduced by Jerome S. Harris), Durham, North Carolina.

Previous investigators have shown that mixtures of equal quantities of normal human gastric juice and 1 per cent casein solution when incubated at 37.5° and pH 7.4 would result in progressive increases in the nitrogenous substances in trichloroacetic filtrates of such digests. This activity was found to be absent, or greatly diminished, in the gastric secretion of patients with Addisonian pernicious anemia.

Since the macrocytic anemia of sprue is indistinguishable in many aspects from that of pernicious anemia, it was decided to investigate the *in vitro* activity of gastric juice from sprue patients. The gastric juice was collected following histamine stimulation. Specimens containing traces of bile-stained duodenal contents were discarded. The gastric juice was adjusted to pH 7.4 and mixed with an equal volume of a 1 per cent solution of sodium caseinate, or egg albumin. The mixture was adjusted to pH 7.4 and set in a constant temperature environment of 37.5° C. for 24 hours. Samples were removed at certain intervals for 24 hours and analyzed for progressive increase in nitrogen as well as increase in amino nitrogen, using the methods previously described.

Four cases of sprue in relapse showed proteolytic activity which was reduced in degree below the normal controls. Six cases of sprue in remission produced results similar to the relapse cases. The proteolysis observed, though reduced below normal values, significantly exceeded results seen in pernicious anemia.

The Modifying Effect of Inorganic Iodine Administered to Thyrotoxic Patients Previously Treated with RAI¹³¹.

A. S. FREEDBERG and ROBERT BUKA (Introduced by J. E. F. Riseman), Boston, Massachusetts.

Considerable disagreement exists as to the advisability of administering stable iodine after the administration of therapeutic doses of I¹³¹ to thyrotoxic patients. This question is of considerable practical as well as theoretic moment. The effect of continued administration of stable iodine begun one or three days after a dose of I¹³¹ was studied in five patients with thyrotoxicosis. Twenty-four hour urinary I¹³¹ excretion, external thyroid Geiger-Muller counts, and in some cases serum radioactivity were determined for periods of ten to thirty days. Comparable studies were carried out in thyrotoxic patients to

whom stable iodine was not given. Clinical evaluation and serial measurements of basal metabolic rate, serum cholesterol, and circulation time were carried out.

The administration of stable iodine begun twenty-four hours after a dose of I^{131} was associated with a marked increase in urinary I^{131} excretion and a pronounced fall in thyroid radioactivity. The administration of Lugol's solution ten minims daily or saturated solution of potassium iodide five minims daily beginning three days after I^{131} dosage was accompanied by a small increase in urinary I^{131} excretion and no appreciable decrease in thyroid radioactivity.

It would appear, therefore, that the early therapeutic effects of stable iodine may be gained by administering it three or more days after I^{131} without interfering significantly with the therapeutic effects of the radioactive material.

The Hemodynamic Effects of Veratrum Viride in Hypertensive Man: Studies of Arterial Pressure, Cardiac Output, Renal and Hepatic Clearances, Peripheral Blood Flow, "Venous Tone" and Vasomotor Reflexes. EDWARD D. FREIS, JOSEPH R. STANTON, JAMES W. CULBERTSON, JULIUS LITTER and MEYER H. HALPERIN (Introduced by Chester S. Keefer), Boston, Massachusetts.

Therapeutic trials of veratrum viride in hypertensive patients have been sufficiently promising in this clinic to stimulate a more extensive investigation of its cardiovascular effects.

Following subtoxic doses of veratrum the fall in arterial pressure (Hamilton) was the result of a decrease in peripheral resistance rather than in cardiac output (direct Fick). Blood flow in the kidneys (PAH), liver (BSP) and limbs (plethysmogram) decreased during the falling phase but then returned to or above control levels during the stable phase of the lowered arterial pressure. "Venous tone" in the limbs decreased but collapse from venous pooling or orthostasis did not occur.

There was no evidence that the hypotensive effects were either sympatholytic or parasympathomimetic. Sympathetic vasomotor reflexes, including vasopressor responses, and digital volume and temperature reactions remained intact. Atropine abolished the bradycardia but not the hypotension after veratrum, while epinephrine or ephedrine abolished both.

Glomerular filtration measured by mannitol clearance was markedly reduced, but by inulin was only transiently decreased. There was always a striking antidiuresis, even without a significant fall in arterial pressure, producing marked increases in the u/P ratio of PAH, mannitol, and inulin.

The Serum Concentration of a Digitalis Glycoside and its Rate of Disappearance in Patients After Parenteral Digitalization. MEYER FRIEDMAN and (by invitation) RENE BINE, JR., San Francisco, California.

By employment of the embryonic duck heart preparation (1), it was found possible not only to detect digitalis

glycoside (Lanatoside C.) in the serum of patients receiving the drug but also to study its rate of disappearance from serum. After preliminary studies had been made of the effects of (1) human blood cells and (2) serum upon the physiological activity of the glycoside, quantitative determinations were made of the glycoside content of the serum of five cardiac patients who had received 1.6 mgm. of Lanatoside C. by vein.

Approximately 0.21 microgram of glycoside per cc. of serum was present in the five patients immediately after its administration. However, the average serum concentration fell rapidly to 0.12, 0.08 and 0.06 micrograms per cc., $7\frac{1}{2}$, 15 and $22\frac{1}{2}$ minutes after injection of the drug. At the end of 30 minutes, the serum of three patients contained 0.05 microgram or less per cc. and in the remaining two patients, no glycoside could be detected in the serum. At the end of an hour, glycoside could not be detected in the serum of any patient. Serum samples taken two, 12 and 24 hours after injection of glycoside also contained no detectable glycoside.

Observations on Intracavitary Electrocardiograms in Man. ARTHUR J. GEIGER and (by invitation) ALLAN V. N. GOODYER, New Haven, Connecticut.

Intracavitary potentials were recorded via a lead passed intravenously in ten subjects including normals and cases of right and left branch block and ventricular hypertrophy.

The changes in P from negative to diphasic to positive as the electrode passed from the superior cava through the atrium into the inferior cava or right ventricle were consistent with the dipole theory of depolarization of muscle. The RS configuration of the normal ventricular complex recorded within the right ventricle was in accord with the view that the septum is depolarized from left to right, assuming that the initial ventricular deflection is of septal origin. Support for this assumption was seen in the entirely negative (QS) character of the ventricular complex in left branch block.

Ventricular extrasystoles excited by the electrode tip in the right ventricle of a case of left branch block closely resembled the natural complexes of the subject.

However, in a case of typical right branch block the triphasic ventricular deflections were of QRs character, implying either that septal depolarization was not from left to right, as ordinarily postulated, or that depolarization of the free wall of the left ventricle preceded depolarization of the septum.

The Effect of Polysaccharides on Virus Activity. (By invitation) HAROLD S. GINSENG, (by invitation) WALTHER F. GOEBEL, and FRANK L. HORSEFALL, JR., New York, N. Y.

Capsular polysaccharides of Friedländer bacilli inhibit multiplication of mumps virus in the allantoic sac of the chick embryo; as little as 5 μ g. of polysaccharide is effective. Inhibition of multiplication is not due to inactivation of the virus *per se* as shown by both *in vitro* and *in vivo* methods. Polysaccharides active as inhibitors

do not block adsorption of mumps virus by cells of the living allantoic membrane. Inhibition of multiplication is obtained when polysaccharide is injected as long as 4 days after inoculation of mumps virus. Chemical studies on the polysaccharide have shown that the structural configurations responsible for specific serological activity are distinct from those which determine the inhibitory effect relative to mumps virus. The available data indicate that polysaccharide acts upon some intracellular system of the host cells in order to inhibit viral multiplication. Moreover, in addition to the evidence obtained from experiments with polysaccharides and viruses, that obtained in interference experiments suggests that viruses of influenza A, B and Newcastle disease require for multiplication host metabolic systems different from mumps virus or pneumonia virus of mice.

The Study of Myocardial Metabolism and Coronary Blood Flow by Coronary Sinus Catheterization. W. T. GOODALE, J. E. ECKENHOFF, R. J. BING, M. LUBIN, J. H. HAFKENSCHIEL, M. H. HARMEL, W. G. BANFIELD, E. L. FOLTZ and S. S. KETY (Introduced by E. Cowles Andrus), Baltimore, Maryland.

A systematic technique of coronary sinus catheterization has been developed in intact lightly anesthetized dogs, without opening the chest, guided by landmarks visible fluoroscopically in the right anterior oblique view. A modified intravenous catheter, having a small tapered tip with multiple openings, has overcome many of the difficulties of insertion and blood sampling in the coronary sinus, avoiding also any undesirable coronary venous obstruction as indicated by pulse pressure recordings. Diodrast coronary venograms showed the orientation of the catheter and the coronary sinus venous system *in vivo*. The nitrous oxide method of Kety and Schmidt, applied to the coronary circulation by catheter technique, gave coronary blood flow values of 70 to 100 cc./100 Gm./min. Myocardial oxygen consumption and carbon dioxide production were 10 to 12 cc./100 Gm./min., or about 10 per cent of the total oxygen consumption, with coronary A-V differences which were 2 to 3 times the overall systemic differences. Left ventricular efficiency varied from 14 to 21 per cent. Myocardial oxygen consumption was well correlated with blood pressure, but poorly correlated with cardiac output.

A very high myocardial utilization of lactic and pyruvic acid was consistently found, even at basal arterial levels, but only a low and inconsistent glucose utilization. The mean lactate/pyruvate ratio was 7 in both arterial and coronary venous blood. The lactic and pyruvic acid uptake together accounted for 40 to 60 per cent of the total cardiac oxygen utilization, while glucose uptake accounted for an average of only 20 per cent.

Endocardial damage could not be entirely avoided in dogs, after catheterization of not only the coronary sinus, but also the pulmonary artery, although technical refinements have minimized the occurrence and size of lesions. Peculiar to coronary sinus catheterization, however, were coronary venous thromboses or myocardial hemorrhages

which sometimes followed prolonged insertion of a catheter far into the sinus or a cardiac vein, with elevated pulse pressures of 9 to 20 mm. Hg which indicated significant coronary venous obstruction. Coronary venous and myocardial damage were avoided by precautions, including gentle insertion of a small catheter only 1-2 cm. inside the coronary sinus. In this position, there was no evident admixture of coronary blood samples with auricular blood, evidence of trauma to the auricle or coronary sinus ostium was minimal, and pulse pressures were the same of only slightly higher than auricular pressures, indicating that there was no significant coronary sinus obstruction by the catheter. Similar precautions are probably advisable in further applications of this procedure.

Observations on the Impedance Plethysmograph. ALLAN V. N. GOODYER (Introduced by David M. Kydd), New Haven, Connecticut.

An impedance plethysmograph has been constructed following the basic design of Dubois and Nims, except for several modifications which were found necessary in order to achieve reproducible calibration of pulse volume changes in terms of resistance units, using an electrocardiograph as the final recording instrument.

Simultaneous tracings obtained from the fingers and arm with this instrument and with the Burch-Winsor plethysmograph were very similar with regard both to wave contours and to calculated absolute pulse volume changes responsible for the deflections. The versatility of the instrument, claimed by previous workers, was confirmed and its range of sensitivity and ease of application proved a decided advantage over the Burch-Winsor apparatus.

Over 100 records from normal subjects and hospital patients have outlined the range and reproducibility of normal pulse patterns obtained from the digits, extremities and trunk, and have indicated certain variations which may be produced by abnormal cardio-vascular states.

The Relationship of the Precordial Electrocardiogram to the Electrical Field of the Heart. ROBERT P. GRANT, (Introduced by Arthur J. Merrill), Atlanta, Georgia.

The precordial QRS and T have been generally assumed to represent principally the electrical activity of that part of the myocardium directly beneath the exploring electrode. The following experiments were designed to study this relationship.

From precordial V-leads taken along numerous vertical axes on the chest, the pathways of the transitional QRS and T complexes around the chest were determined. Then, in the same subject, the mean spatial vectors representing the size and directions of the forces producing the QRS and T were determined by an adaptation of Wilson's tetrahedral method. The pathways of null points which these spatial vectors would produce on a cylinder of the same dimensions as the patient's chest were then computed. The precordial V-lead transitional pathways were found to coincide closely in all characteristics with the path-

ways calculated for the electrical field of the whole in the eight cases studied. The method was then simplified for routine use and in over 300 consecutive normal and abnormal subjects reasonable agreement was found.

It is concluded that the precordial deflections represent the electrical activity of the heart as a whole, as far as direction of deflection is concerned. Accordingly the distribution of positive and negative precordial QRS and T waves is a function of the directions of the spatial QRS and T vectors. Since these vectors are computed from limb leads, it is evident that the precordial deflections are also functions of the ventricular gradient. By studying this relationship of the precordials to the vectors as manifested in the limb leads the interpretation of abnormal and unusual precordial patterns is much simplified and clarified.

The Uptake of Radioactive Phosphorus by Gastric Carcinoma. (By invitation) SEYMOUR J. GRAY, (by invitation) JOHN SCHULMAN, JR. and (by invitation) MARLENE FALKENHEIM (Introduced by Clifford L. Derrick), Boston, Massachusetts.

Tracer doses of radioactive phosphorus were injected into patients with carcinoma of the stomach and in patients with nonmalignant gastric disease approximately 36 hours prior to gastric resection. The cancerous gastric mucosa and the non-cancer gastric mucosa were analyzed for total phosphorus, acid soluble phosphorus and nucleoprotein content. The radioactivity of each constituent was determined. Results were expressed as standard specific activity [per cent of (injected dose per gm. of body weight) per mgm. of phosphorus]. This expression is proportional to the rate of turnover of phosphorus in the tissue.

In non-cancer stomachs the turnover of phosphorus was uniform. No differences were observed between those areas where cancer is prone to develop and where benign lesions are usually found. In cancerous stomachs the phosphorus turnover of the non-cancerous mucosa was the same as the normal stomach. The turnover of total phosphorus of the carcinoma was 47 per cent greater than the non-cancer mucosa. The turnover of phospholipid phosphorus was 45 per cent greater and the nucleoprotein phosphorus turnover was 56 per cent greater in the cancer than in non-cancer tissue.

Inhibition of Multiplication of Influenza Virus by Tannic Acid. ROBERT H. GREEN (Introduced by Francis G. Blake), New Haven, Connecticut.

Recently several reports describing the inhibition of multiplication of certain viruses have appeared. In some instances the substances which inhibit virus multiplication also inhibit virus hemagglutination. Furthermore, some of these substances are themselves capable of agglutinating erythrocytes. The interrelationships among these phenomena, if indeed any exist, are not clear.

During the course of studies on hemagglutination it was found that tannic acid in concentrations as low as

45 μ g. per cc. agglutinates chicken erythrocytes. Further investigations revealed that tannic acid, in dilutions higher than those which produce hemagglutination, actually inhibits the agglutination of chicken erythrocytes by influenza A virus. Concentrations ranging from 5 to 20 μ g. per cc. inhibit virus hemagglutination. Moreover, tannic acid inhibits the multiplication of influenza A virus *in vivo* and inactivates the virus *in vitro*. When injected into the chorio-allantoic sacs of embryonated eggs one mg. per egg markedly inhibits the multiplication of virus, and smaller amounts produce a less marked but appreciable degree of inhibition. The inhibitory effects of tannic acid would appear to be due, at least partly, to a direct action upon the virus because, *in vitro*, very low concentrations of tannic acid inactivate large amounts of virus.

Right Auricular Pressures in Man at Rest and During Exercise. DAVID G. GREENE, CHARLES E. ROH and ELEANOR DEFOREST BALDWIN (Introduced by Franklin M. Hanger), New York, N. Y.

Subjects with normal cardiac function and subjects with varying degrees of congestive failure were studied by means of right heart catheterization at rest and during supine exercise. Right auricular pressures were measured with Hamilton manometers. The cardiac output was determined by the direct Fick method.

In subjects with normal cardiac function, who responded to the exercise with a definite increase in cardiac output, no significant rise in right auricular mean pressure was observed. On the other hand, in patients with impaired cardiac reserve exercise was associated with a rise in right auricular mean pressure irrespective of any change in the cardiac output. The magnitude of auricular systole and of the negative wave associated with the descent of the base was greater during exercise than at rest in some cases of each group.

Results of Treatment of Patients with Hypertension by Total Thoracic and Partial to Total Lumbar Sympathectomy, Splanchnicectomy and Celiac Ganglionectomy. K. S. GRIMSON and (by invitation) E. S. ORGAIN, Durham, North Carolina.

During the last 8 years 108 patients have been treated by subtotal to total sympathectomy. This operation differs from others currently employed for hypertension in that it includes removal of the stellate and upper thoracic ganglia and therefore denervates the head, arms and thorax as well as the splanchnic visceral area. Usually, only sympathetic pathways to the legs are left intact. Operative mortality occurred in four patients. Two patients died three and five days after reduction of pressure with respiratory arrest; one patient died with uremia and one died with myocardial infarction. At present eleven patients have died since operation. Deaths with three exceptions were caused by sudden cardiac or cerebral vascular accidents. No patient has developed uremia since operation. Varying degrees of clinical improvement or apparent cure have been achieved in the 93 patients

now alive. Supine blood pressure was reduced to normal in 25, reduced but not to normal in 44, and not reduced in 24. Postural hypotension has occurred and has persisted with the exception of a few patients who years after sympathectomy have systolic but not diastolic postural lowering of pressure. Bradycardia has developed after sympathetic heart denervation and has persisted. Retinal hemorrhages and exudates or papilledema were present in more than a third of the patients before operation. With few exceptions these disappeared and have not recurred. Generally symptoms of hypertension have disappeared. Other significant clinical data will be presented and interpreted.

4-Caproylamino Diphenylsulfone, 4'-Aminomethylsulfonic Acid Sodium Salt. Pharmacology and Effect in Experimental Tuberculosis. RICHARD GUBNER, RENÉ J. DUBOS, CYNTHIA PIERCE and HARRY E. UNGERLEIDER (Introduced by William Dock), Brooklyn, New York.

The sodium salt of 4-caproylamino diphenylsulfone, 4'-aminomethylsulfonic acid (Equityl) has been synthesized in an attempt to find a sulfone compound of low toxicity possessing chemotherapeutic activity against the tubercle bacillus. The *in vitro* bacteriostatic effect of this compound is identical with that of diaminodiphenylsulfone, with complete inhibition of growth of human strains of the tubercle bacillus in drug concentrations of one to two mgms. per cent in the Dubos medium. Unlike diaminodiphenylsulfone Equityl possesses no bacteriostatic effect against the streptococcus, staphylococcus, pneumococcus or diphtheria bacillus in concentrations up to 100 mgms. per cent.

The drug exhibits important pharmacological differences from diaminodiphenylsulfone. Whereas diaminodiphenylsulfone given orally to mice causes fatal toxicity in single dosage of 0.25 to 0.5 G./kg. no toxic effects whatever are observed on administering as much as 4 G./kg. of Equityl in single dosage by stomach tube. In chronic toxicity studies in mice no toxic effects were observed when the drug was given daily for thirty-eight days in dosage of 0.25 G./kg., or when given for twenty-one days in dosage of 0.67 G./kg. Similarly in man there has been no evidence of toxicity on oral administration in single dosage of 5 Grams or on protracted daily dosage of 2 to 4 Grams up to ten weeks, as judged by symptoms, weight, blood counts, icteric index, blood chemistry and urinalysis. By the parenteral route, however, Equityl exhibits the same toxicity in mice as diaminodiphenylsulfone. The reason for the lack of toxicity on oral administration appears to be limited absorption of the drug despite its high solubility, for regardless of the oral dosage blood levels above 3.8 mgm. per cent have not been observed. Following a single 5 G. dose in man the drug is rapidly absorbed to its maximum blood level within one hour and is bound to the serum proteins. It is excreted slowly; during the first twenty-four hours urinary concentrations up to 25 mgms. per cent are obtained, with a total twenty-four urinary excretion of approximately 200 milligrams, urinary excretion thereafter falls off

gradually over several days. These pharmacological properties are very similar to findings previously reported by one of us with another caproyl compound, *i.e.*, N_4 -caproylsulfanilhydroxamide.

The chemotherapeutic effect of Equityl has been investigated in experimental mouse tuberculosis. 0.02 mgm. (dry weight) of a virulent 10-day old H37 strain of tubercle bacillus was inoculated in the tail vein of young Swiss mice. Equityl was mixed with the feed beginning on the day of inoculation in concentrations of 0.25 mgm. per cent and 0.075 mgm. per cent, representing a daily dosage of approximately 0.67 G./kg. and 0.25 G./kg. respectively. All of the control group of ten animals died within fourteen days, with a weight loss averaging 4.3 G. Gross pulmonary tuberculous lesions were present, of the mixed pneumonic, granulomatous, and caseating type. The treated animals were sacrificed after twenty-one days, within which time one death, not due to tuberculosis, had occurred. Pulmonary lesions of minimal degree were present in the majority of the treated animals, less so in the group receiving the larger dosage of drug. In the larger dosage group no weight loss occurred whereas in the smaller dosage group there was an average weight loss of 3.7 G. Equally effective protection could be obtained with diaminodiphenylsulfone although the margin between the therapeutic and toxic dosage was found to be very narrow.

The conclusion is drawn that Equityl possesses chemotherapeutic activity against the tubercle bacillus comparable to diaminodiphenylsulfone, with the advantage of complete freedom from toxicity on oral administration.

Studies on the Mechanism of Hemolytic Anemia and Hemoglobinuria Occurring in Patients with High Concentrations of Serum Cold Agglutinins. THOMAS HALE HAM and (by invitation) FRANK H. GARDNER, (by invitation) PHILIP F. WAGLEY, and (by invitation) S. C. Shen, Boston, Massachusetts.

Patients have been observed with hemolytic anemia associated with high concentrations, in the serum, of cold autohemagglutinins. Studies of mechanical fragility of blood samples containing cold agglutinins, at 15°, 11°, and 4° C., showed increasing hemolysis of normal red cells and red cells from the patients proportional to the increasing degree of agglutination observed at these temperatures. The increased mechanical fragility, observed *in vitro*, on chilled blood containing cold agglutinins, suggested that mechanical trauma to the agglutinated red cells in the periphery of the body might be one of the mechanisms of destruction of red cells in patients with hemolytic anemia associated with cold agglutinins.

In one patient with chronic acquired hemolytic jaundice of 12 years' duration, there was continued high concentration of cold agglutinins (1-5000) observed over a period of four years. The red cells showed a normal osmotic fragility, but a strongly positive agglutination with anti-human serum rabbit serum (Coombs test), and an abnormally increased mechanical fragility at 37° C. without agglutination. The mechanical

fragility was significantly increased at 15° C. Chilling the arm for 20 minutes at 15° with or without stasis, in observations made on two occasions 4 years apart, produced on both occasions hemoglobinemia and hemoglobinuria. At 37° C., using the same procedures, no hemoglobinuria was produced. A similar study was conducted on a patient without anemia who was recovering from atypical virus pneumonia, associated with a high concentration of cold agglutinins (1-5000) in the serum. The red cells were normal as evidenced by normal osmotic and mechanical fragilities and negative Coombs test. Chilling of the arm at 15° C., at which temperature there was a significant increase in mechanical fragility, did not produce hemoglobinemia. Accordingly, hemoglobinemia and hemoglobinuria appeared to result from mechanical destruction *in vivo* of agglutinated red cells during chilling at 15° C., but only in the patient with red cells that were manifestly abnormal.

Three additional cases were observed with high concentrations of cold agglutinins in the serum associated with increased osmotic fragility of the red cells, and, in two instances, with spherocytosis. In one of these patients, there was extreme hemoglobinuria without exposure to cold. The mechanism for these changes is not known.

The Action of Iodocasein on Human Myxedema, with Comparative Studies on the Fate and Distribution of Synthetic Radioactive Iodocasein and of I^{131} during Hypothyroidism and Euthyroidism. (By invitation) C. FERRILL HAMILTON, (by invitation) A. ALBERT, (by invitation) MARSCHELLE H. POWER, (by invitation) SAMUEL F. HAINES and F. RAYMOND KEATING, JR., Rochester, Minnesota.

Tracer doses of I^{131} and of synthetic radio-iodocasein, comparable in total iodine and specific radioactivity, were administered before and after myxedema in a patient was completely alleviated according to conventional criteria by daily administration of 60 mg. iodocasein. This dose was equivalent by bio-assay to 100 mg. strong thyroid. The fate and distribution of I^{131} and radio-iodocasein were determined by radioactive measurements of urine, feces and blood, and by *in vivo* measurements over thigh, liver and thyroid areas.

During myxedema, I^{131} was distributed in urine (91 per cent), feces (2 per cent) and in blood, where an appreciable fraction was protein-bound. Thyroid and liver areas showed no concentration of radioactivity. After administration of radio-iodocasein, however, urine contained 40 per cent of the radioactivity, feces 59 per cent and blood only small amounts, most of which was protein-bound. Considerable radioactivity was concentrated only over the liver. When euthyroidism was attained, repetition of each tracer yielded qualitatively similar results respectively but the proportion of protein-bound radioactivity in blood was diminished.

These studies indicate that (1) intermediary metabolism of organic iodine may occur in the liver and (2) human myxedema can be corrected by synthetic iodocasein.

Arterial Pressure Response to the Valsalva Test: an Indicator of Sympathetic Activity. ESTHER HARDENBERGH, JAMES L. WHITTENBERGER and STANLEY J. SARNOFF (Introduced by David D. Rutstein), Boston, Massachusetts.

Changes in femoral arterial pressure resulting from a simulated Valsalva experiment (extrathoracic and intrapulmonary pressures of 40 mm. Hg for 30 seconds) have been observed 357 times in 10 dogs. Measurements have been made with an electronic strain gauge, direct writing oscillograph, and reveal the following:

(1) The response consists of six components (A, B, C, D, E, F).

(2) Component E, the overshoot of arterial pressure above the prestimulus level following cessation of the stimulus, is of major importance because: (a) it is consistently present in normal and vagotomized animals; (b) it is diminished in direct proportion to the degree of interference with sympathetic activity accomplished by graded segmental spinal anesthesia or tetraethylammonium chloride; (c) it is increased by elimination of vagal activity accomplished by vagotomy or the administration of atropine; (d) it is greatly diminished or abolished by interference with carotid sinus activity accomplished by previous bilateral occlusion of both common carotid arteries.

These results explain the mechanism of the arterial pressure response to the Valsalva test and indicate the basis for its use clinically in the determination of the extent of sympathetic activity and denervation.

Experiments with Pteroylglutamic Acid and Pteroylglutamic Acid Deficiency in Human Leukemia. ROBERT W. HEINLE and (by invitation) ARNOLD D. WELCH, Cleveland, Ohio.

Administration of pteroylglutamic acid (PGA) to three patients with chronic myeloid leukemia was attended by rapid hematologic and clinical relapse in each instance. In one of these patients, hematologic improvement accompanied the withdrawal of PGA on two occasions. Administration of PGA to patients with chronic lymphoid leukemia was not associated with relapse. One patient with chronic myeloid leukemia was placed on a regimen of diet low in PGA, a crude antagonist of PGA, and succinylsulfathiazole. After one hundred days, marked hematologic remission with drop of leukocyte count from 150,000 to 12,000 per c.mm. occurred with concomitant decrease in platelet count from normal values to 10,000 per c.mm. Upon subsequent administration of PGA and withdrawal of the antagonist, relapse occurred and the patient died, although reinstitution of the original regimen was followed by evidence of hematologic improvement just before death. With the rise in white count there was also an increase in platelet count to 150,000 per c.mm. While these experiments are not conclusive, they indicate the desirability of further study of the role of pteroylglutamic acid in white cell genesis in general and leukemia in particular.

Pulmonary Capillary Pressure in Man. (By invitation)

HARPER K. HELLEMS, (by invitation) FLORENCE W. HAYNES, (by invitation) JOHN F. GOWDEY and LEWIS DEXTER, Boston, Massachusetts.

A cardiac catheter with the hole on the tip has been introduced into a small branch of the pulmonary artery so as to obstruct the arterial lumen and pressures have been recorded with Hamilton and saline manometers. The pressure existing in the lumen of the artery distal to the obstruction is a result of the retrograde transmission of pressure from the next significant collateral branch entering the pulmonary artery. Anatomically, this is the pulmonary capillary bed of the lung. Physiologically, this is also the case, as indicated by the fact that blood fully saturated with oxygen can be withdrawn through the catheter. Furthermore, pressures recorded through catheters wedged into the pulmonary artery and pulmonary vein are in essential agreement.

In normal individuals the pulmonary capillary pressure, thus measured, varies from 7 to 15 mm. Hg with a mean average of 10. In emphysema, pulmonary vascular disease, Eisenmenger's Complex, with elevated pulmonary artery pressure, the pulmonary capillary pressure is within normal limits, indicating that the resistance to blood flow is in pulmonary "arterioles" and not in the capillaries. In mitral stenosis and other cardiac abnormalities producing so-called left heart failure, the pulmonary capillary pressure is elevated at rest and increases further during exercise.

The Sertoli Cell. CARL G. HELLER, and (by invitation)

WILLIAM O. MADDOCK, (by invitation) EDWIN O. JUNGCK, and (by invitation) WARREN O. NELSON, Portland, Oregon.

In each of 32 patients having azoospermia or oligospermia, with or without eunuchoidism, it was noted that some seminiferous tubules were devoid of all cells of the germinal series, revealing only the supporting cells of Sertoli. The percentage of tubules containing Sertoli cells only varied from 10 to 100 per cent, and were usually associated with some degree of thickening or hyalinization of the tunica propria. Varying degrees of germinal activity were encountered, from instances in which the majority of tubules contained germinal cells to instances (5) in which 100 per cent of the tubules contained Sertoli cells only. This suggests that the condition of "Sertoli cells only" is the result of death of the germinal cells.

The clinical appearance of these patients, varying from completely eunuchoidal to normal, could be correlated with the histological appearance of the Leydig cells, which varied from severe degeneration to normal.

The Sertoli cell does not appear to secrete a hormone capable of inhibiting the secretion of pituitary gonadotrophins, since urinary gonadotrophins appeared in greater than normal amounts in the urine of each patient.

Lack of inactivation of follicle stimulating hormone due to lack of spermatogenic activity is suggested as the

factor accounting for the rise in urinary gonadotrophin excretion.

Procaine Penicillin: an Experimental and Clinical Evaluation. WALLACE E. HERRELL, and (by invitation) DONALD R. NICHOLS and (by invitation) FORDYCE R. HEILMAN, Rochester, Minnesota.

This study concerns itself with the efficacy of procaine penicillin in prolonging the action of this antibiotic. Various types of suspensions of procaine penicillin have been studied. These include procaine penicillin in cottonseed oil, in peanut oil and in sesame oil, as well as aqueous suspensions. After single intramuscular injections of 1 cc. of the material (300,000 units per cubic centimeter) adequate therapeutic blood levels are maintained for twenty-four hours or longer. In some instances therapeutic levels of penicillin in the serum have been maintained for as long as forty-two hours following a single injection.

The report includes observations on the pharmacologic action of this material, including absorption, excretion and diffusion.

The therapeutic results obtained in the treatment of a variety of infections owing to microbes susceptible to the action of penicillin are also included in the report.

Postural Changes in Cardiac Output in Orthostatic Hypotension. JOHN B. HICKAM (Introduced by J. M. Ruffin), Durham, North Carolina.

The fall of blood pressure in postural hypotension may be explained by lack of normal arterial constriction or by a greater than normal postural fall in cardiac output. Failure of arterial constriction in response to a fall of blood pressure has been found in cases of postural hypotension, but there is little information on variation in cardiac output with change of body positions.

Cardiac outputs were measured by the Fick method, and intracardiac and peripheral arterial pressures were manometrically recorded. Determinations were made in the supine and semi-erect positions on 4 cases of idiopathic postural hypotension and in 5 patients with postural hypotension produced by lumbo-dorsal sympathectomy for hypertension.

In 4 partially sympathectomized patients there was a large drop in blood pressure in the foot-down position, but the cardiac output showed no more than the normal postural fall. In 3 cases of non-operative postural hypotension and in one sympathectomized patient there was a postural fall in both cardiac output and mean arterial pressure to approximately 50 per cent of the supine values. One patient who frequently had postural hypotension showed, at the time of study, only a negligible fall in blood pressure but a 50 per cent drop in cardiac output in the foot-down position. In the cases of non-operative postural hypotension and in one sympathectomized patient a rapid infusion of albumin in saline prevented more than a normal postural fall in cardiac output and blood pressure.

It is concluded that a large drop in cardiac output, as

well as failure of compensatory arterial constriction, is a factor in the postural fall in blood pressure in certain cases of orthostatic hypotension. The mechanism of the drop in cardiac output is unexplained. The restoration of the output and the blood pressure on acutely increasing the blood volume suggests that in certain patients with postural hypotension failure of normal venoconstriction may play a part.

The Effect of a Nitrogen Mustard on Whole Blood Coagulation Time. L. O. JACOBSON, (by invitation) J. G. ALLEN, (by invitation) T. R. SMITH, (by invitation) C. L. SPURR, and (by invitation) M. H. BLOCK, Chicago, Illinois.

Allen and Jacobson recently reported that ionizing radiations produced a prolonged whole blood coagulation time in humans and animals as a result of the appearance in the blood of an anticoagulant biologically indistinguishable from heparin. This phenomenon was also observed in five patients with neoplastic disease given therapeutic doses of a nitrogen mustard (methyl bis (B chloroethyl) amine hydrochloride). A prolonged whole blood coagulation time has also been produced in rabbits by the intracardial injection of this drug in a dose of 3 and 4 mg. per kilogram of body weight. In both humans and rabbits a pancytopenia, prolonged bleeding time, and prolonged whole blood coagulation time (Lee White) are associated with the syndrome, whereas the prothrombin time is normal.

Usefulness of "Gamma Globulin" Determinations in Estimating Duration of "Activity" in Streptococcal Infections and in Rheumatic Fever. B. V. JAGER, J. F. WALDO and H. H. HECHT (Introduced by Maxwell M. Wintrobe), Salt Lake City, Utah.

In an attempt to find a more sensitive index of persistent activity in streptococcal infections and in rheumatic fever than is afforded by the erythrocyte sedimentation rate, a simple chemical method has been devised for measuring "gamma globulin."

Repeated clinical and laboratory examinations have been carried out in 15 patients with beta hemolytic streptococcal pharyngitis at intervals over a period of 6 weeks to 1 year. Five patients showed clinical evidence of non-suppurative complications. In these 5 and in 3 others without clinical findings, an elevation of the "gamma globulin" was demonstrable for prolonged periods, often in the absence of other abnormal laboratory findings.

In 15 patients with acute rheumatic fever, repeated studies have been made for a period of 6 to 18 months. In this group, the "gamma globulin" fraction was frequently greater than normal in the absence of other abnormal findings. Moreover in some patients whose infection was judged inactive, cyclical recurrent rises occurred in this fraction, suggesting persistence of the process at a less intense level. Such activity, ordinarily unrecognized, may account for the progressive cardiac changes which are so apt to occur long after the rheumatic attack has apparently subsided.

"Gamma globulin" and antistreptolysin O titer are being measured periodically in normal individuals with the object of determining whether by this means subclinical streptococcal infections can be detected.

Studies on Dicumarol in Human Beings: Its Neutralization by Vitamin K₁ Oxide, Menadiol Bisulfite, Synkayvite and Blood. DAVID F. JAMES, JOHN J. BUTLER, IVAN L. BENNETT, JR., and PERITZ SCHEINBERG (Introduced by Marshall N. Fulton), Atlanta, Georgia.

In order to establish a broader clinical basis for the control of dicumarol effect, vitamin K₁ oxide, menadiol bisulfite and synkayvite were administered in large single doses to patients with hypoprothrombinemia induced by dicumarol. The effect of fresh and bank blood transfusions was evaluated. One hundred and ten patients were given dicumarol in the usual dosage. When the prothrombin time exceeded that of normal plasma diluted to 20 per cent the patient was either allowed to return to his normal prothrombin level or was given large single doses of the test substances. Blood samples taken frequently during the succeeding 24 hours, and daily thereafter, were studied for prothrombin activity.

The efficacy of these substances was tested on two bases: the time elapsing until (1) the conversion of marked to moderate hypoprothrombinemia and (2) the appearance of a prothrombin level consistent with the possibility of intravascular clotting. Vitamin K₁ oxide was markedly superior in both respects. Patients given 0.5 gram or more of this material intravenously required an average time of 4 hours to be changed from that of marked to moderate hypoprothrombinemia. An average of 13 hours elapsed until the appearance of a prothrombin level consistent with intravascular clotting.

Bank and fresh blood were equally effective, 500 cc. transfusions having a minor, temporary effect.

The requirement of an individual for dicumarol is approximately predictable from his prior response to this agent. An exception to this situation occurs shortly after the administration of vitamin K₁ oxide, following which patients are relatively insensitive to dicumarol. This signifies storage of vitamin K₁ oxide for several days.

Studies on Prolonged Suppurative Infection in Man. Observations on the Blood. (By invitation) GEORGE W. JAMES, III, (by invitation) LILLIAN A. RIBLET, (by invitation) JOSEPH C. ROBINSON, (by invitation) ROBERT E. JOHNSON, and ROBERT M. KARK, Chicago, Illinois.

Eighty-six young men with chronic suppurative infections of the bones, kidneys or soft tissues were studied by clinical and laboratory techniques. Despite vigorous therapy with blood and plasma transfusions, sulfonamide derivatives and antibiotics, chronic suppuration persisted with frequent breakdowns. When first observed infection was severe in eleven and moderate in the remainder. The average duration of illness was 24 months and the average loss of weight was 7.9 kilograms. No clinical evidence of vitamin or protein deficiencies existed.

Distribution curves for plasma proteins, hemoglobin, hematocrit, white and red cells were within normal limits. Slight deviations from the normal were noted in the M.C.H.C., and M.C.V. The red cells of patients with severe infection showed decreased osmotic resistance. Serum iron concentration was diminished with increased serum copper concentration, especially when severe infections existed.

Blood volume per kilogram body weight was normal in 13 patients with moderate, and in ten with severe, infection. In the latter total circulating hemoglobin was reduced despite transfusions, while circulating plasma protein levels were maintained or increased even in the face of a marked reduction in body mass (from 14 to 24 per cent). Periodic determinations of serum copper and iron made in the severely ill showed a return to normal levels concomitant with clinical improvement. The shift in copper came earlier and was more marked than the shift in iron.

Observations on the Bone Marrow of Persons with Chronic Hepatic Insufficiency and Macrocytic Anemia.
(By invitation) THOMAS JARROLD and RICHARD W. VILTER, Cincinnati, Ohio.

Macrocytic anemia commonly observed in persons with chronic liver disease has been linked in theory to the erythrocyte maturation factor deficiency anemias. This relationship has not been tested by studies of bone marrow morphology and there are several reasons for questioning it. We wish to report studies on the peripheral blood and bone marrow performed repeatedly in twenty unselected patients with advanced portal cirrhosis confirmed in the majority of instances by biopsy or autopsy.

Moderate or severe anemia, usually macrocytic and normochromic, was found in seventeen subjects. Blood smears revealed slight anisocytosis and poikilocytosis, and normal differential nucleated cell counts. Reticulocytosis up to 13 per cent was common and could not be explained by recent blood loss in the majority of patients.

The bone marrow was hypo- or normally cellular with a relative increase in normoblasts. Megaloblasts and early erythroblasts were found in only two subjects both of whom had good evidence of extrinsic factor deficiency.

Plasma cells and lymphocytes were found in strikingly increased numbers, many times as high as 20 per cent for each cell type. The degree of plasma cell and lymphocyte hyperplasia correlated roughly with the degree of hyperglobulinemia suggesting a causal relationship.

In no instance did intensive therapy with liver extract, folic acid, amino acids or B complex vitamins affect the peripheral blood or bone marrow dramatically. Additional reticulocytosis did not occur.

These data suggest that a metabolic abnormality other than lack of storage or utilization of the erythrocyte maturation factor must account for the macrocytic anemia in chronic liver disease. In an occasional patient, extrinsic factor deficiency may superimpose a hematologic pattern resembling pernicious anemia.

Radio-active and Stable Iodine in Peripheral Tissues.

MACALISTER W. JOHNSTON (Introduced by William T. Salter), New Haven, Connecticut.

It is well established that the protein-precipitable iodine of plasma reflects thyroid activity. A similar correlation can be demonstrated with peripheral tissue iodine when the inorganic fraction is separated from the organically bound. Studies in rats and cats show a species difference in iodine concentrations; but both yield values lower than normal in hypothyroidism (produced by thiouracil) for muscle, liver, kidney, heart and brain. Higher values than normal are found in animals treated with thyroxine.

The organically bound iodine of peripheral tissues (*e.g.*, skeletal muscle) can be isolated in association with certain characteristic protein fractions.

In observations involving man and animals, when measurement of radio-activity is combined with classical studies of stable (ordinary) iodine, the resulting ratio (*i.e.*, "specific radio-activity") indicates the rate of metabolic turnover under appropriate conditions. The same comparative procedure can be applied to the thyroid gland and blood plasma.

After treatment with radio-active iodine, peripheral tissues also contain radio-active iodide (inorganic). If this be present for appropriate periods of time at high concentrations, tissue damage will result.

The Renal Tubular Reabsorption of Salt with Exercise in a Patient with Cardiac Failure and Normal Controls.

A. KATTUS, B. SINCLAIR-SMITH, J. GENEST and E. V. NEWMAN (Introduced by A. M. Harvey), Baltimore, Maryland.

Simultaneous clearances (C) of inulin (In), para-amino-acetylhippuric acid (PACA), chloride, sodium, potassium and phosphate were determined on three occasions in a young patient with congestive failure due to rheumatic valvular disease during mild exercise. On normal subjects the effect of walking at different rates on simultaneous clearances of inulin or creatinine (C_r), and electrolytes was observed. The patient at rest had a low normal C_{In} with an abnormally high filtration fraction (FF). Exercise caused a fall in C_{In} with no change in FF and a marked fall in ratios C_{Na}/C_{In} and C_{Cl}/C_{In} . The ratio C_K/C_{In} fell slightly with urine flow and C_{In} . During recovery, C_{In} returned to control level before the C_{Na}/C_{In} and C_{Cl}/C_{In} ratios.

Normal subjects walking showed unchanged C_{In} or C_{Cr} , but the ratios C_{Na}/C_{In} and C_{Cl}/C_{In} decreased consistently and sometimes as markedly as in the cardiac patient. The ratios C_K/C_{In} and C_{HPO_4}/C_{In} fell slightly with the urine flow.

Thus, increased renal sodium chloride retention is a normal response to exercise due to increased tubular reabsorption of filtered electrolytes, not necessarily related to fall in filtration rate, but possibly elicited more readily and intensely in cardiac failure.

The Significance of Aortic and Pulmonary Artery Wall Movements, Electrocardiographically Recorded, in the Study of Acute Circulatory Disturbances. CALVIN F. KAY, (by invitation) JAMES W. WOODS, JR., and (by invitation) HARRY F. ZINSSER, Philadelphia, Pennsylvania.

Border movements of the aorta and pulmonary artery were continuously recorded by the electrocardiograph in 18 normal subjects during the period of acute change in intrathoracic pressure produced by voluntary straining. Brachial artery pressure was simultaneously recorded with a capacitance monometer and, in several subjects, stroke volume was estimated with the ballistocardiograph. Pulsation amplitude changes recorded from the aortic knob were directly related to changes in brachial pulse pressure and inversely related to the pulse rate. In pulsations recorded from the pulmonary artery, a striking increase in amplitude immediately followed cessation of straining, as observed in pressure recordings from this vessel by others. We employed procedures to estimate the method as a measure of the movements of the vessel walls and to estimate the relation of vessel wall movements to diameter changes. It is concluded that the electrocardiographic method provides a measure of relative stroke change in aortic and pulmonary artery diameter. From the electrocardiographic records alone, the cardiovascular disturbances produced by straining may be deduced. The interpretation so obtained is in accordance with that previously derived by the combined application of many methods. It is a rapid, simple, and painless method by which cardiovascular dynamic changes are immediately and sharply recorded. It is now proving useful in the study of a variety of cardiovascular problems.

The Cerebral Circulation in Essential Hypertension and the Effects of Differential Spinal Sympathetic Block. (By invitation) SEYMOUR S. KETY, (by invitation) BENTON D. KING, (by invitation) JOSEPH H. HAFKENSCHIEL, (by invitation) STEVEN M. HORVATH, and WILLIAM A. JEFFERS, Philadelphia, Pennsylvania.

Quantitative measurements of cerebral blood flow, cerebrovascular resistance and cerebral oxygen consumption have been made in a series of patients with essential hypertension by means of the nitrous oxide technique. Cerebral blood flow and oxygen consumption are within normal limits, but there is a striking increase in cerebrovascular resistance. Because of the frequency of cerebral symptoms following the acute reduction of blood pressure in this condition it was deemed of interest to perform these quantitative observations during a period of relative hypotension produced by differential spinal block. A fall in mean arterial blood pressure was obtained, accompanied by a significant reduction in cerebral blood flow. There was no change in cerebral oxygen consumption or in cerebrovascular resistance. The cerebral nutritive index (ratio of oxygen supplied to oxygen consumed), determined independently of cerebral blood flow, showed a moderate decrease during the hypotension and a striking reduction where the blood pressure fell markedly and

cerebral symptoms became apparent. All changes reported are statistically significant.

These findings indicate that the high degree of vascular tone present in the brain in essential hypertension is not readily released even in response to a fall in blood pressure of a severity sufficient to compromise the cerebral blood flow.

Studies in Methionine and Sulfur Metabolism in the Presence of Liver Damage: I. Rate of Utilization and Urinary Excretion of the D and L Isomers Following Intravenous Administration. LAURANCE W. KINSELL, HAROLD A. HARPER and HARRY C. BARTON (Introduced by Theodore L. Althausen), San Francisco, California.

It was postulated that intravenously administered methionine would be anabolized and catabolized at a different rate in patients with liver damage than in normal humans.

To test this hypothesis, 1.5 grams of DL-methionine has been administered to normal individuals, and to patients with acute and chronic liver damage. Plasma L and DL-methionine, urinary L and DL-methionine, and urinary inorganic and ethereal sulfate have been determined before, and at frequent intervals following the methionine infusion. Microbiologic assay procedures have been utilized for methionine quantitation.

Such studies to date have demonstrated:

1. Significant diminution in the rate of methionine utilization in patients with liver damage, as compared to normals, with reversion to normal as liver function improves.

2. Insignificant urinary L-methionine, but very considerable D-methionine excretion.

3. Urinary inorganic sulfate excretion appears to give significant information as to the relative anabolism or catabolism of administered methionine. A persistent catabolic response may be a grave prognostic sign.

4. Studies in methionine-sulfur metabolism in other metabolic disorders will be presented if time permits.

Vitamin A Studies in Middleaged and Old Individuals. ESBEN KIRK and MARGARET CHIEFFI (Introduced by William B. Kountz), Saint Louis, Missouri.

An investigation was made of the total carotene, α & β carotenes, and vitamin A concentration of plasma in 155 middleaged and old individuals, offered a diet adequate in vitamin A, and in 47 younger subjects. The vitamin A analyses were performed on 20 ml. plasma samples, using a modification of the technique recommended by the Association of Vitamin Chemists. The total carotene concentration was obtained by use of a Dubosque colorimeter, the α & β carotene value after preliminary adsorption to an aluminum oxide column and subsequent elution with benzol-benzene. Definitely higher total carotene and α & β carotene concentrations were found in the younger (16-39) age group (average values 330 and 190 micrograms per cent) than in the middleaged and old individuals (average values 210 and 110 micrograms

per cent). The vitamin A concentration of plasma averaged 20 micrograms per cent and showed no significant change with age.

In the patients presenting a low plasma vitamin A value (1-15 micrograms per cent) and the incidence of conjunctival pathology and of toad skin was significantly higher than in the individuals in whom a high (30-60 micrograms per cent) vitamin A plasma value was found. No certain difference was observed in the daily output of epithelial cells in the urine or in the percentage frequency of cornified cells in conjunctival smears. The evaluation of the dark adaptation values was rendered difficult by the presence of lenticular opacities and retinal arteriosclerosis.

The Relation of Growth Dispersion to Growth Inhibition of M. Tuberculosis by Subtilin and Other Chemotherapeutic Agents. VERNON KNIGHT and RALPH TOMPSETT (Introduced by Walsh McDermott), New York, New York.

A preparation of subtilin highly inhibitory to pneumococci, streptococci, and tubercle bacilli *in vitro*, was also shown to possess high *in vivo* activity in experimental pneumococcal and streptococcal infections. It was further observed that under suitable experimental conditions, persistence of antimicrobial activity against tubercle bacilli in the serum of treated animals could be demonstrated by biologic assay. Nevertheless, the course of tuberculous infection in mice was not inhibited by the continued administration of subtilin in daily doses which were much larger than those necessary for protection against pneumococcal or streptococcal infections.

Investigation of the mechanism of this paradoxical phenomenon revealed that it is associated with an unusual property of *M. tuberculosis*. In contrast with most other disease-producing bacterial species, *M. tuberculosis* grows *in vitro* as macroscopic aggregates, unless non-ionic wetting agents are added to the medium. It was observed that tubercle bacilli growing as aggregates were not inhibited by subtilin. Conversely, when the addition of wetting agents converted growth to the dispersed form characteristic of other bacteria, the cells of *M. tuberculosis* were highly sensitive to minute concentrations of subtilin, as well as to certain other chemotherapeutic agents. Presence of the same wetting agents in diffusely growing cultures of other bacterial species produced no appreciable effect upon the usual sensitivity of the cells to subtilin or other antimicrobial drugs. It was also observed that when tween, one agent used for dispersion of growth, was antagonized by serum, the tubercle bacilli grew in aggregates and were not inhibited by subtilin. This effect of serum could be neutralized by the addition of sufficient wetting agent to permit dispersed growth.

It is not known whether the aggregates of tubercle bacilli present *in vivo* are in any way comparable in their drug sensitivity to the aggregates which occur *in vitro*. It was observed, however, that the few chemotherapeutic agents which are anti-tuberculous *in vivo*, are highly active against the aggregated organisms growing *in vitro*

and display relatively little increase in activity when tested under conditions of dispersed growth.

Dipolar Nature and Duration of the Regression Process in the Human Heart. CHARLES E. KOSSMANN, New York, N. Y.

In records made of the action potential in the heart of any given species under physiologic conditions, the deflections resulting from regression are partly obscured by the larger and more rapid deflections resulting from accession. By warming the sinus venosus of the frog, it has been possible to separate the two sets of deflections, and to demonstrate that the former, like the latter, are dipolar in nature, but of reversed polarity and approximately twenty times greater length (Macleod, 1938).

In two different sets of observations in man similar features of the regression process were demonstrated in the right atrium, and in the ventricles.

In the first of these observations an electrogram was made after introducing a small exploring electrode into the cavity of the right atrium of a patient with sinus rhythm and complete auriculoventricular block. Deflections of atrial recovery could be observed without distortion by oscillations of ventricular excitation. The entire auricular complex was composed of an initial positive and negative deflection followed by a slower pair of waves of lower voltage and reversed polarity (Figure 1). From the record, the tripartite nature of the early part of the action potential was easily discerned. The duration of increasing activity, disregarding the error introduced by the distance of the electrode from the atrial endocardium, was 0.005 second; of complete activity 0.0028 second; and of receding activity 0.075 second. Assuming the rate of conduction in the human atrium to be approximately 2000 mm. per second (Kossmann, *et al.*, 1947), the length of accession was 10 mm., of complete activity 56 mm., and of regression 150 mm.

In addition, a terminal, slow, small positive deflection, comparable to the U wave in ventricular records, was observed.

At necropsy sometime later, the right atrium was found to be greatly dilated, so that the figures cannot be accepted as representative of the normal.

In a second set of observations electrocardiograms were made in patients whose body temperature was purposefully reduced to approximately 80° F., by cooling in air. In addition to other changes (Kossmann, 1939), several patients displayed an unusual ventricular T wave consisting of two parts, both upright, one of which occurred just after the QRS, the other in the usual location at the end of electrical systole (Figure 2). The first of these was in the expected direction but the second was not, presumably because of a ventricular gradient.

The two experiments were interpreted to mean that in man, as in lower species, the regression process in the auricles and in the ventricles is essentially dipolar in nature, and of considerably greater length than the accession process.

The Motility of the Esophagus in Cardiospasm and Scleroderma. (By invitation) PHILIP KRAMER and FRANZ J. INGELFINGER, Boston, Massachusetts.

Esophageal motility was studied by fluoroscopy and balloon-kymograph recordings in normal subjects, 3 patients with cardiospasm and 4 patients with scleroderma.

In both cardiospasm and scleroderma cases, fluoroscopy demonstrated definite but not excessive esophageal dilatation ending abruptly near the cardia. In scleroderma, motility was minimal. In cardiospasm, motor activity was present, but uncoordinated and non-propulsive. Though degree of stasis in patients with cardiospasm and scleroderma was comparable, esophageal symptoms were minimal in scleroderma cases.

Motility records revealed marked atonicity and diminished wave pattern in both cardiospasm and scleroderma. Acetyl-beta-methyl-choline, 10 mg. i.m., increased motility moderately in normal subjects, exerted little effect on the hypomotility of scleroderma, but produced a tetanic and lumen-obliterating contraction of the esophagus in cardiospasm. This reaction was also observed fluoroscopically.

These observations support the concept that cardiospasm is a neuromuscular disorder of the entire esophagus, not of the cardia alone. The violent esophageal reaction to cholinergic stimulation may be the hypersensitive response of a denervated organ and suggests that the distal parasympathetic pathways are interrupted in cardiospasm. Though superficially alike, the esophageal disorders of cardiospasm and scleroderma do not appear similar with respect to pathogenesis or function.

The Relation between Infant Birthweight and Subsequent Development of Maternal Diabetes Mellitus. (By invitation) JOSEPH P. KRISS and PALMER H. FUTCHER, Saint Louis, Missouri.

Analysis was made of birthweight data on infants born to 100 women destined later to develop diabetes and on infants born to 100 non-diabetic (control) women of comparable age and parity. In the 2 groups there were 144 infants weighing 10 pounds or more; 77.1 per cent of the 144 were born to pre-diabetic women. Of 52 infants weighing 12 pounds or more, 90.3 per cent were born to pre-diabetic women. Single and repeated births of abnormally large infants occurred far more commonly in the pre-diabetic mothers than in the control mothers. As reported by H. C. Miller, the average birthweight of the infants born to the pre-diabetic mothers was significantly greater than that of infants born to control mothers. The period between the birth of the first abnormally large infant and the development of clinical diabetes in the mother averaged about 24 years, with a range from 1 to 46 years.

According to Spiegelman and Marks, it may be predicted that 4 per cent of women of childbearing age will develop diabetes. The data on our small group of subjects indicated that diabetes developed in over 50 per cent of women giving birth to one baby weighing over 13 pounds or to 3 babies weighing more than 10 pounds.

Experimental Studies on Spread of Pain. E. CHARLES KUNKLE, GEORGE C. ARMISTEAD and HELEN GOODELL (Introduced by Harold G. Wolff), New York, New York.

In 125 experiments upon 23 adult subjects cyclic pain induced by immersion of a finger in water maintained at 0° C. was almost always found to overflow to adjacent areas, particularly to neighboring digits. Features common to this phenomenon were a "latent" period, "facilitation" in subsequent phases of the pain cycle, "tapering" of intensity, "incomplete segmental filling," and absence of contralateral spread. Pain failed to spread from thumb to jaw (in cortical sequence). Overflow of pain was unaltered by preliminary interruption of the circulation to the arm or by procainization of an area into which spread of pain was to occur.

The extent of spread showed moderate intra- and marked inter-individual variation. In a minority of instances the spreading pain "migrated" during the experiment, "skipped" a digit, or reached a higher intensity than that of the primary pain.

These listed features can readily be identified also in clinical experience, notably in patients with angina pectoris. It is inferred that such overflow of pain is a central phenomenon and probably occurs at the segmental level in the cord. This mechanism contrasts sharply with spread of pain due to peripheral effects, as in secondary contraction of skeletal muscle or with sensory nerve root or trunk lesions.

The Metabolic Effects of Chorionic Gonadotrophins in Young Men. RICHARD L. LANDAU and KATHRYN KNOWLTON (Introduced by Allan T. Kenyon), Chicago, Illinois.

The anabolic effects of testosterone are established but it has not been shown that the testis of the mature male is capable of participating in anabolic adjustments by an increased rate of secretion of its hormone. To study this possibility chorionic gonadotrophins were administered to two normal men and one eunuchoid.

One normal man received 500 to 3000 I.U. of chorionic gonadotrophins daily for six days. Pronounced nitrogen retention of 60 mg./Kg. body weight daily was obtained. There was parallel retention of inorganic phosphorus and administered creatine. 17-ketosteroids rose an average of 7 mg. daily for ten days. Another received 500 to 1500 I.U. daily for twelve days. Nitrogen retention (22 mg./Kg. daily) was moderate, as was that of phosphorus. 17-ketosteroids showed only a faint suggestion of a rise.

The eunuchoid was given 500 to 3000 I.U. daily for six days without effect on the urinary constituents determined. However, 5 mg. of testosterone propionate daily produced nitrogen retention of 28 mg./Kg. per day. The effects of the gonadotrophins described are accordingly mediated through the testis.

The normal adult testis is thus sensitive to suitable stimulation.

The Relation of Renal to Non-renal Vascular Resistances in "Essential" Hypertension; and the Effect of Sympathectomy. (By invitation) M. LANDOWNE, A. S. ALVING and (by invitation) W. ADAMS, Chicago, Illinois.

Studies of renal dynamics were made upon twelve subjects before and up to twenty-eight months after radical sympathectomy. In nine of these, non-simultaneous cardiac output was determined under comparable basal conditions. For simultaneous measurements the ratio of renal to non-renal blood flow would be $RBF/(CO - RBF)$, and the ratio of renal to non-renal resistances (R_k/R_n) would be approximately the reciprocal of this. For non-simultaneous measurements this ratio (R_k/R_n) has been derived from the calculated renal resistance (R_k) and the total resistance (R); since in parallel arrangement

$$\frac{1}{R} = \frac{1}{R_n} + \frac{1}{R_k},$$

therefore

$$\frac{R_k}{R_n} = \frac{R_k}{R} - 1.$$

Renal resistances were calculated by Lampport's formulae, using the sum of "afferent" and "efferent Arteriole" resistances. Total resistances were expressed, in the same units, as $P_m/CO \times 1000$.

The average of the preoperative ratios R_k/R_n was 4.27. Twenty-nine postoperative determinations gave an averaged value of 4.17. The result of sympathectomy upon the blood pressure is not the same in all patients. In those of our series with lower R_k 's ($R_{a+s} < 32$ units/ $1.73 m^2$) the blood pressure fell. Two of the nine cases were in this group; their preoperative R_k/R_n was 2.85, and postoperatively fell to 1.42. Five cases had temporary reduction in blood pressure after operation. Their averaged R_k/R_n was 4.00 before, and 3.83 after operation. Two cases did poorly. In these the preoperative R_k/R_n was 6.4, and the postoperative R_k/R_n was 7.8. The significance and limitations of the data will be discussed.

Physiological Studies of Polycythemia Vera. JOHN H. LAWRENCE, and (by invitation) R. LOWRY DOBSON, (by invitation) WM. E. BERG, (by invitation) LOUIS R. WASSERMAN, (by invitation) JAMES R. ROBERTSON, and (by invitation) ROBERT L. ROSENTHAL, Berkeley, California.

A method employing a mass spectrocope for analysis of expired gas mixtures was used to measure the rate of pulmonary denitrogenation, taken as a measure of pulmonary efficiency. As the patient changed from breathing air to inhaling pure oxygen a continuous record of nitrogen concentration in exhaled air was continuously recorded. No marked difference was found between polycythemia patients and normal controls in the group studied.

Using the microgasometric technique of Roughton and Scholander, arterial blood oxygen saturation was found to be within normal limits in 51 resting patients with polycythemia.

Determinations of prothrombin, bleeding and clotting times and studies of clot retraction by electrical resistance methods in polycythemic patients gave normal values.

Arm-to-tongue circulation times and blood viscosity were found to be elevated in patients having high hematocrits. In general they fell to normal as the hematocrits approached normal with treatment.

Sternal marrow studies showed the characteristic pattern for polycythemia vera to be a relative increase in nucleated red cells.

Using a thermal conductivity method, it was found that after exercise polycythemic patients have a normal rate of return to normal of oxygen consumption but an abnormally slow return to normal of CO_2 production.

The Incidence of Reaction Following Administration of Crystalline Aqueous Penicillin, Penicillin in Oil and Beeswax and Procaine Penicillin in Oil. (By invitation) MARK H. LEPPER, HARRY F. DOWLING, (by invitation) JAY A. ROBINSON, and (by invitation) THOMAS E. STONE.

This study reports the relative incidence of local and allergic reactions resulting from use of aqueous penicillin in peanut oil and beeswax (POB) and procaine penicillin in sesame oil (PPO).

The incidence of local reactions recorded as mild, moderate and severe was obtained. During 317 courses of treatment, 232 patients receiving aqueous penicillin, there were 27 mild, one moderate and two severe reactions. Similarly, during 272 courses of therapy with POB in 170 patients similar reactions occurred in 64, 21 and 9 patients respectively. With PPO there were 72 courses and 54 patients with only 4 reactions, all mild. With all preparations there was a greater incidence of reaction with higher doses and the difference is greatest in the POB treated group. With the aqueous and POB preparations the number of reactions increased with longer administration, but this trend has not been seen as yet in the PPO patients. There is a comparable percentage of patients receiving high doses and long continued treatment with each preparation. POB is more apt to cause local reactions and the dosage and duration of use are limited by this fact.

In evaluating allergic reactions, many of the patients were given a second course of the same preparation. However, the reaction rate is very small, there being two such reactions in the aqueous, one in the POB, and none in the PPO patients discussed above.

The Physiologic Activity of Tetrabrom- and Tetrachlor-thyronine. JACOB LERMAN and (by invitation) C. R. HARRINGTON, Boston, Massachusetts.

One of the actions of thyroid hormone is to depress the function of the thyroid. This is accomplished in two ways: 1. Indirectly by depressing pituitary activity, and 2. Directly by depressing the thyroid follicle. Advantage has been taken of this property to depress the overactive thyroid in Graves' Disease. However, the high calorigenic action of thyroid hormone makes it hazardous to

use it in large doses over a long period of time. The desired compound is one which has little or no calorigenic activity and yet may retain the property of depressing the thyroid.

Two such compounds have been made available by Harington, namely tetrabromthyronine and tetrachlorthyronine. Each compound has been assayed in two patients with spontaneous myxedema, and the results compared with our standard assays of thyroxine and thyroxinopoly-peptide. In each case, there was a rise in metabolism, improvement in the myxedematous state and reduction in serum cholesterol. That iodine did not participate in the metabolic effect produced by tetrabrom- and tetrachlorthyronine is indicated by the fact that the protein-bound iodine of the blood remained unchanged. The activity of tetrabromthyronine is about 1/15th, and of tetrachlorthyronine about 1/300th that of thyroxin.

A preliminary trial with tetrabromthyronine in a patient with Graves' Disease caused a slight drop in metabolism but a marked drop in protein-bound iodine from 24 γ to 8.8 γ per cent.

Studies of Phosphorus Metabolism in Man: II. A Study of the Permeability of the Human Erythrocyte to Inorganic Phosphate in Vitro and in Vivo by the Use of Radioactive Phosphate (P^{32}). S. M. LEVENSON, M. A. ADAMS and F. H. L. TAYLOR (with the technical assistance of Mary Kendrick) (Introduced by George Richards Minot), Boston, Massachusetts.

Phosphate exchange between the plasma and erythrocytes of human blood was studied *in vitro* and *in vivo*. The *in vitro* studies were conducted over a period of 4 hours. In both instances P^{32} was used as a means of tagging the phosphorus. In the *in vitro* studies, unlike former studies on this subject, no phosphate was added other than the isotopic preparation which was of high specific activity.

The *in vitro* studies showed that inorganic phosphate exchanged freely between plasma and erythrocytes at 37.5° C. Minimal transfer occurred at 7° C.

Essentially all the P^{32} in the plasma remained in the inorganic form. Most of the P^{32} which passed into the erythrocyte was found in the inorganic fraction, less than 30 per cent of the amount found in the erythrocyte after 4 hours being in the organic form. The transfer to the organic fraction was confined entirely to the acid soluble portion.

Following the intravenous administration of tracer amounts (100 to 200 microcuries) of P^{32} in man, the exchange and distribution of the isotope followed closely those observed *in vitro*.

The Study of Hemoglobin Metabolism in Man with the Aid of the Isotope Technique. IRVING M. LONDON, DAVID SHEMIN and D. RITTENBERG (Introduced by R. West), New York, New York.

The administration of glycine labeled with N^{14} to humans affords a physiologic method for studying the rate

of formation and the pattern of destruction of the human erythrocyte. In three normal adults the average life span of the erythrocyte has been found to be 127, 116, and 118 days. A patient with sickle cell anemia has shown a random disappearance of labeled heme from the peripheral blood which is consistent with either a random destruction of the erythrocytes or a random synthesis and degradation of hemoglobin in the peripheral blood. In investigating this question, the whole blood of sickle cell anemia patients has been found to synthesize heme from glycine *in vitro*. Blood from normal individuals and from patients with elevated reticulocyte counts due to other blood dyscrasias produced no significant heme synthesis. These studies will be considered in relation to our earlier studies in pernicious anemia and polycythemia vera.

Crystalline stercobilin has been isolated from the feces of one of the normal subjects in order to study the relationship of hemoglobin destruction to bile pigment production. The data indicate that a portion of bile pigment is derived from a source other than the hemoglobin of circulating red blood cells. The origin of this portion of bile pigment will be discussed.

The Pathogenesis and Histopathology of Air-Borne Pneumonitis Virus Infection in Mice: The Effect of Penicillin G upon the Developing Lesion. CLAYTON G. LOOSLI and (by invitation) MERLE H. RITTER, Chicago, Illinois.

Fatal pulmonary infections were produced in mice by allowing them to breathe air for 1 hour in a 60-liter chamber into which was atomized 4 cc. of 10^{-3} dilution of mouse-lung suspension of mouse pneumonitis virus which is related to the psittacosis-lymphogranuloma group. Animals died of extensive pulmonary consolidation from 10 to 16 days following exposure to the infected atmosphere. The development of the pulmonary lesion was studied in mice killed at increasing intervals after exposure. Grossly small focal lesions appeared on lung surfaces in from 3 to 4 days. These enlarged by directed extension until the greater majority of the lung substance became consolidated when death occurred. There was evidence microscopically that the virus "central body" and "plaque" develop extracellularly on the surface of the alveolar walls, an observation which is in agreement with that of Weiss and others. The intracellular virus vesicle is confined principally to the attached septal cells of the alveolar walls. Daily injections of Penicillin G (1000 units) given subcutaneously in 4 doses prevents the growth of the primary pulmonary lesion. The effect of the penicillin on the developing virus bodies in the lung will be shown.

Induced Insulin Resistance in the Rabbit. FRANCIS C. LOWELL and (by invitation) WILLIAM FRANKLIN, Boston, Massachusetts.

A number of rabbits, which were shown to be susceptible to small doses of insulin, were injected with

large doses of beef and pork insulin incorporated in an emulsion of falba and mineral oil containing acid-fast organisms. Four months after injections were begun one animal developed a high degree of insulin resistance which persisted for at least 5 months. A single intravenous dose of 16 units of insulin, equivalent to 400 units in a human subject, was tolerated without symptoms and the blood sugar fell only moderately. On the other hand small doses of *human* insulin have caused a pronounced fall in blood sugar. Diabetes has not developed and a glucose tolerance test was normal. The resistance to insulin, therefore, is species specific and is probably due to the development of an antibody for beef and pork insulin.

Further experiments have shown that beef insulin treated chemically so as to destroy its activity as a hormone, still acted as though it combined with the animal's antibody for insulin. It is concluded that resistance to insulin on an immunologic basis may be induced in the rabbit, that such resistance may be species specific and that the antibody formed is not a true anti-hormone. It appears that experimentally induced resistance to insulin may provide a valuable immune system for experimentation as well as a new means for studying insulin. Finally, it is probable that these findings have a bearing on resistance to insulin as it occasionally occurs in diabetic individuals.

The Relationship between the Plasma Protein Level, the Renal Excretion of Sodium, and Edema. JOHN A. LUETSCHER, JR., and (by invitation) ALASTAIR D. HALL, Baltimore, Maryland.

The anomalous behavior of certain nephrotic patients after albumin therapy suggested that renal retention of sodium might be as important a cause of edema as the reduced plasma colloid osmotic pressure. Both increased circulating protein and diuresis of sodium and water are apparently necessary to raise the concentration of plasma protein to normal, but either factor alone may increase the plasma protein concentration to a limited extent. The plasma volume rises when circulating protein is increased, but decreases during diuresis.

In acute hemorrhagic nephritis, edema may be associated with renal sodium retention without significant reduction in the plasma proteins, and diuresis may result in a sharp drop in plasma volume with increasing concentration of the circulating proteins.

Dogs on protein-free diets for 3-4 months show a profound fall in glomerular filtration rate and some impairment of sodium excretion. Specific depletion of plasma protein to similar levels by plasmapheresis is associated with minimal reduction in filtration rate and sodium excretion.

These data suggest that either protein depletion or failure of renal sodium excretion may lower the plasma protein concentration. When the two factors coincide in nephrosis, intractable hypoproteinemia and edema follow. The specific sodium retention may be attributed to active nephritis or to prolonged protein deficiency.

Observations on the Dumping Syndrome and Relief of Symptoms by Atropine. THOMAS E. MACHELLA, Philadelphia, Pennsylvania.

In some patients following subtotal gastric resection, symptoms of varying severity occur after the ingestion of meals; these may consist of sweating, vertigo, palpitation, weakness, nausea and even collapse. It is generally accepted that the rapid entrance of food into the jejunum is somehow responsible for the manifestations. The mechanism of their production has been variously ascribed to mechanical distention of the jejunum, to hypoglycemia and also to hyperglycemia.

Observations on 4 patients manifesting symptoms of the "dumping syndrome" reveal that the symptoms occur toward the end of a meal or immediately thereafter during which period a hyperglycemia exists. They are accompanied by a rise in blood pressure and an increase in pulse rate. The manifestations can be reproduced by the administration of glucose orally but not when the glucose is administered intravenously. The symptoms, but not the rise in blood sugar, in blood pressure or in pulse rate, are prevented by the administration of atropine before meals. The results of mechanical distention of the jejunum by inflation of a balloon will be presented.

Observations on the Apparent Acquisition of Streptomycin-Fastness. MANSON MEADS (Introduced by George T. Harrell, Jr.), Winston-Salem, North Carolina.

Methods of preventing drug-fastness in clinical infections are suggested by observations made on strains of *Kl. pneumoniae* exposed to streptomycin. When the number of organisms and the antibiotic concentration were varied independently, in liquid or solid media, a small number of progeny of originally sensitive cells grew in streptomycin. This number was inversely related to the drug concentration up to a point where the population was large. A constant number of highly resistant cells then appeared. Variants surviving small drug concentrations gave rise to variants of greater streptomycin resistance. Sulfonamide and penicillin sensitivities were unaffected. Variants occurred only during active cell division. Resistant variants are reported to appear following exposure of bacteria to sulfonamides, bacteriophage, penicillin, and gramicidin.

A rapidly developing high degree of streptomycin resistance occurs more frequently, clinically, than a step-like slowly developing resistance. Gram negative pathogens tend to focalize and produce large populations. The frequency of drug-fastness should be reduced if: (1) the number of infecting organisms can be reduced prior to specific therapy; (2) *bacteriostatic* drug concentrations are maintained in the infected site; and (3) another specific bacteriostatic drug, or antiserum, is used concurrently. The second drug should retard growth of the few variants of high resistance specifically to streptomycin.

A New Approach to the Vascular Problem in Diabetes Mellitus. (By invitation) R. MEGIBOW, (by invitation) S. MEGIBOW, (by invitation) K. OSSERMAN, (by invitation) J. BOOKMAN, (by invitation) S. FEITELBERG, and HERBERT POLLACK, New York, New York.

A new direct writing photoelectric microplethysmograph capable of recording volume changes of magnitudes less than 1 mm.³ has been employed to obtain plethysmograms from the great toes of 53 diabetic patients ranging from 6 to 66 years in age.

Plethysmographically the presence of structural vascular disease is manifested by a reduction in peripheral blood flow as measured by the venous occlusion method, and by a reduction in the amplitude of the volume pulse waves after the release of vasomotor tone by nitroglycerine and tetraethylammonium. Since blood flow through these vessels represents the ultimate circulation, the combination of a normal oscillometric index and of an abnormal plethysmogram, if present, would suggest that the initial vascular alterations in diabetes develop in the arterioles and capillaries.

Such a combination of clinically unsuspected but plethysmographically demonstrated vascular disease was noted in 10 of 53 patients. In an attempt to elucidate what factors might initiate these capillary and arteriolar changes, the plethysmograms were correlated with the age of the patient, the duration of the diabetes, the type of diet, the dosage of insulin, the adequacy of control, and the level of the blood cholesterol. The results are discussed, and the implications from both the therapeutic and pathogenetic viewpoints are stressed.

The Effect of Anemia and Polycythemia on Digital Intravascular Blood Viscosity. MILTON MENDLOWITZ (Introduced by Louis J. Soffer), New York, New York.

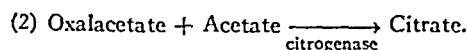
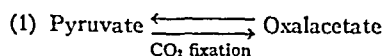
The calorimetric method for measuring digital blood flow was used to study changes in intravascular blood viscosity with varying erythrocyte concentration in patients with anemia and polycythemia. In each observation the blood vessels were dilated maximally by indirect heating. Change in the ratio of pressure to flow with increasing erythrocyte concentration was used as a measure of change in viscosity, the factor of vascular caliber having been maintained relatively constant. Pressures were corrected for "yield value" which varied with erythrocyte concentration. Flow values were corrected for the specific heat of the blood which also varied with erythrocyte concentration. The final observations confirmed similar studies in animals, indicating that intravascular blood viscosity differs from *in vitro* measurements. The gradient of decrease in viscosity with decreasing erythrocyte concentration in anemia was moderate. The blood viscosity in polycythemia increased gradually with moderate increase in erythrocyte concentration and more steeply at higher levels. The blood viscosity was found to be 80 per cent of normal at a hematocrit level of 17 and 169 per cent of normal at

a hematocrit level of 73, these being the extremes of anemia and polycythemia observed.

Studies on Pyruvate and Citrate Metabolism in Man and Animal. MAX MILLER, (by invitation) ERNEST BUEDELING, and (by invitation) R. O. STRAUCH, Cleveland, Ohio.

Few of the individual steps of intermediary metabolism have been demonstrated in the intact animal. Consequently, some of the metabolic reactions involving glucose, alanine, citrate, succinate, acetate, and pyruvate were studied *in vivo*. Blood pyruvate rose from 50 to 375 per cent following citrate, glucose, alanine, and succinate injections, the substances being arranged according to increasing effect. Acetate initially caused a fall in pyruvate, with a subsequent rise. Simultaneous administration of glucose and acetate abolished the initial rise in pyruvate produced by glucose alone, but greater rises than with either occurred after 60 minutes. Plasma citrate fell significantly after glucose and alanine injections, elevations resulted after succinate and acetate.

The rises in pyruvate coincide with observations *in vitro* that pyruvate is an intermediary in the metabolism of glucose, alanine, citrate and succinate. The greater rise after succinate and alanine can be explained by fewer intermediary reactions leading to the formation of pyruvate. The initial fall in pyruvate after acetate is consistent with the hypothesis that a C₂ compound related to acetic acid condenses with oxalacetate formed by CO₂ fixation from pyruvate:



The secondary rise in pyruvate after acetate injection could be due to the subsequent conversion of citrate to pyruvate.

The rises in citrate after succinate and acetate infusions indicate that these compounds are metabolized by the same intermediary reactions (tricarboxylic acid cycle) as *in vitro*. The fall in citrate levels after glucose and alanine cannot be satisfactorily explained by the above schemes and other possible pathways will be discussed.

The Role of "Insulinase" in the Regulation of Carbohydrate Metabolism. I. ARTHUR MIRSKEY and (by invitation) R. H. BROH-KAHN, Cincinnati, Ohio.

In another communication we have described the occurrence, distribution and properties of an enzyme system which is capable of inactivating insulin during *in vitro* incubation and which, for descriptive purposes, we call "insulinase." In order to determine its role in the regulation of carbohydrate metabolism in the intact organism, it seemed desirable to investigate the influence of various procedures on the content of this system in the liver. Toward that end, the insulinase content of the liver of

normal well-fed rats was compared with that of copper treated and fasted animals.

In accordance with the observation that the addition of copper inhibits its activity *in vitro*, the liver insulinase content of rats injected subcutaneously with copper sulphate was found to be significantly lower than that of untreated rats. Further, when rats were fasted for 48 hours or more, their liver insulinase content underwent a significant decrease which could be restored to normal values within 48 hours after cessation of their fast. In many instances, a positive correlation could be established between the insulin sensitivity of an animal and its liver insulinase content. The clinical application of these findings will be discussed.

Oxygen Tension in the Skin of the Extremities. HUGH MONTGOMERY and (by invitation) ORVILLE HORWITZ, Philadelphia, Pennsylvania.

Method: Oxygen tension of the intact skin of toes was derived from measurements made by a modification of the exposed tip electrode method of Davies and Brink. The circuit comprised a sharp platinum electrode, diameter 0.25 mm., a moist indifferent electrode, a calomel half-cell, a galvanometer, and a source of suitable polarizing voltage. When O_2 diffusion coefficient and temperature were kept constant, current varied directly with PO_2 in known solutions. The platinum electrodes were calibrated for use in intact skin by insertion into excised skin in 0.9 per cent NaCl of known PO_2 . Corrections for variations in skin temperature were resolved, and were included in the method.

Results: The work (unpublished) of Hodes and Larabee was confirmed, showing current increasing with warmth of skin, increasing several fold when the subject breathed pure O_2 , and becoming zero when the circulation to the limb was arrested by pressure.

By means of calibrated electrodes, the PO_2 of skin was measured. Skin of normal toes averages 100 mm. Hg. during vasodilatation, 50 mm. during vasoconstriction, and 500 mm. during vasodilatation when the subject breathed pure O_2 . Severely ischaemic skin of toes of patients with arteriosclerosis had PO_2 as low as 5 mm., and little or no increase resulted from the breathing of O_2 .

Methods and Interpretations in the Study of Intracellular Biochemistry by Isotope Dilution Technics. FRANCIS D. MOORE, Boston, Massachusetts.

Fluid phase partition in human patients has been extended by the use of deuterium to measure total body water (H_2^{18}). Total solid elementary constituents may also be measured by isotope dilution; in this study the measurement of the total exchangeable potassium (K_t^{42}) has occupied our attention.

Technical problems, biological limitations and factors which determine interpretation of data derived by these methods, will be discussed relative to the findings on seventy-five hospital patients.

Resistance to the Action of the Endotoxins of Enteric Bacilli in Man. HERBERT R. MORGAN (Introduced by Maxwell Finland), Boston, Massachusetts.

The intravenous injection of 0.001 mg. of purified, toxic, somatic antigens (endotoxins) of *Salmonella typhosa*, *S. schottmuelleri* and *Shigella dysenteriae* in man produced chills, fever, headache, muscle aching and generalized malaise and in some instances nausea and vomiting. Daily injections resulted in a progressive decrease in the severity of these reactions until 5 or more times the original dose could be administered without any reaction. Patients who developed this tolerance to the toxic effects of any of these three antigens following its repeated injection were found to show the same resistance following the injection of either of the other two endotoxins. This tolerance was not related to the presence of specific circulating antibody, since patients becoming resistant to injections of the antigen from *S. typhosa* showed no reaction to the administration of *Sh. dysenteriae* when antibodies were detectable only for *S. typhosa*.

The acquired tolerance disappeared in 4-6 weeks, although homologous antibody was still detectable. Some mechanism other than an antigen-antibody reaction is probably responsible for this phenomenon of tolerance.

A patient convalescent from typhoid fever was resistant to the toxic effects of these somatic antigens.

Muscle Electrolytes in Patients with Potassium Depletion. GILBERT H. MUDGE (Introduced by Robert F. Loeb), New York, New York.

Muscle electrolytes were studied by analysis of biopsies obtained from five patients who showed evidence of potassium depletion. Three patients had chronic renal acidosis and osteomalacia and two had gastric alkalosis. In three of these simultaneous sodium, potassium, chloride and nitrogen balances were studied.

Biopsies showed that intracellular water (calculated as non-chloride water) contained decreased potassium and increased sodium concentrations. These intracellular cation changes were similar in both acidosis and alkalosis. In one patient with chronic renal acidosis the biopsy was obtained when the patient had severe voluntary muscle paralysis. Serum and intracellular potassium concentrations were low but did not differ significantly from the non-paralyzed patients, suggesting that the changes in potassium concentration, in themselves, were not the direct cause of this type of paralysis.

Balance data, calculated by the method of Darrow on the assumption that chloride remains extracellular, demonstrated shifts of sodium and potassium which were in general agreement with the changes found by biopsy, although some inconsistencies were noted.

The Splanchnic Oxygen Consumption of Man in the Normal and Diseased States, with Observations on the Effect of the Intravenous Amino Acids. J. D. MYERS and B. C. HOLLAND (Introduced by Eugene A. Stead, Jr.), Durham, North Carolina.

The simultaneous measurement of the hepatic blood flow and the arterial-hepatic venous oxygen difference, by

the method of catheterization of the hepatic veins, provides an estimation of the splanchnic oxygen consumption (hepatic blood flow \times hepatic A-V oxygen difference). Under conditions of rest and fasting, the splanchnic oxygen consumption, which is the oxygen consumption of the liver plus those viscera drained by the portal vein, is probably reflective of the true hepatic oxygen consumption.

The splanchnic oxygen consumption has been measured under various circumstances in which it might be suspected of being altered—cardiac failure, severe anemia, hyperthyroidism, and after the rapid intravenous administration of amino acid solution. The results are compared with data obtained in a series of 12 individuals without significant disease.

Study of 13 patients with heart failure and low cardiac outputs has shown a *proportionate decrease* in hepatic blood flow, as measured by the bromsulphalein technique. This decrease in flow is compensated by an increase in arterial-hepatic venous oxygen difference so as to maintain a normal splanchnic oxygen consumption. In 5 subjects with hemoglobin levels of less than 8 gm. per 100 ml., all of whom showed increased cardiac outputs, there was a *proportionate rise* in liver blood flow and again a normal splanchnic oxygen consumption. Four patients with hyperthyroidism have shown increases in splanchnic oxygen consumption in keeping with their increases in total oxygen consumption. This was accomplished largely by an increase in hepatic A-V oxygen difference; there has been mild if any increase in hepatic blood flow.

A well-tolerated solution of amino-acids (Vuj-nIX solution, Merck) has been given rapidly intravenously in a dose of 25 to 50 gm. (250 to 500 ml.) to 12 individuals (controls 4, cardiac failure 3, anemia 3, and hyperthyroidism 2). This was accompanied, in all of the subjects except those with anemia, by a striking increase in splanchnic (and presumably hepatic) oxygen consumption. The increase was accomplished primarily by a rise in hepatic A-V oxygen difference. Certain individuals, such as those with heart failure and hyperthyroidism, had such marked unsaturation of hepatic venous blood in the fasting state that not much further increase in oxygen extraction was possible. These persons, then, supported their increased splanchnic oxygen consumptions after amino acids by augmenting the liver blood flow by as much as 50 per cent of the basal.

The Effect of Changing Plasma Concentration on Clearances of Diodrast (C_D), Para-aminohippuric Acid (C_{PAH}) and Para-aminoacetylhippuric Acid (C_{PACA}) in Dog and Man. E. V. NEWMAN, J. GENEST, A. GENEJIN, E. CALKINS and J. MURPHY (Introduced by Benjamin M. Baker, Jr.), Baltimore, Maryland.

Clearances were determined on fasting, resting dogs and patients after single intravenous injections producing continuously falling plasma concentration. In dogs the C_{PAH} and C_{PACA} were constant and identical from 8 to 1.0 mgm. per cent plasma concentration. Renal ex-

traction of $PACA$ was 80–90 per cent, determined from renal vein plasma in an explanted dog kidney.

In man the C_D and C_{PAH} fell progressively as plasma concentration fell from 10 to 1.0 mgm. per cent. The fall in C_{PAH} in man was accompanied by increased proportion of conjugated PAH (C_{CPAH}) in urine. The C_{CPAH} was always higher than the C_{PAH} .

The C_{PACA} in man showed no self depression with plasma concentration up to 7 mgm. per cent and did not show progressive fall with falling plasma concentration.

No de-acetylation of $PACA$ and PAH was found in dog and man, and no conjugated PAH was found in dogs.

The filtration rate was constant as the C_{PAH} and C_D fell in man.

The C_D and C_{PAH} are not independent of falling plasma concentration in man, but the C_{PACA} is; the C_{PAH} and C_{PACA} are independent in the dog.

Hereditary Hypercholesterolemia: A Factor in the Genesis of Coronary Atherosclerosis. Studies of Patients Under Age of 50. ALBERT D. PARETS and DAVID ADLERSBERG (Introduced by Ernst P. Boas), New York, New York.

Study of 122 unselected, consecutive patients with proven coronary artery disease under age of 50; 108 males and 14 females. The average serum cholesterol for the entire group was 316 mgms. per 100 ml. Arcus senilis was exhibited by 22, xanthelasma by 12, and xanthomatosis by 3; the great majority with these stigmata had hypercholesterolemia. Fifty families of these patients were available for studies. In 15 families, all or most of the siblings showed hypercholesterolemia, in 9 families there was an equal number of siblings with normo- and hypercholesterolemia. Only concentrations of serum cholesterol above 300 mgms. per 100 ml. were considered abnormal. The findings suggest that a hereditary disturbance of lipid metabolism may play a significant part at least in young individuals with coronary artery disease.

The Action of Penicillin on Staphylococcus. The Effect of a Short Exposure to Penicillin on Growing Cells. R. F. PARKER, Cleveland, Ohio.

When a growing culture of staphylococcus is exposed to an appropriate concentration of penicillin, growth is promptly inhibited. Earlier work with a single strain indicated that if after a short interval the cells were transferred to penicillin free broth at 37° C. no appreciable killing of organisms occurred. On the other hand, such penicillin treated cells failed to resume growth for a considerable period.

In the present experiments the observations have been extended to include 29 strains, and it has been found that the effect is consistently produced. Experiments indicate that when staphylococci sensitive to inhibition by 0.25 unit of penicillin per ml. in the standard test are exposed for 15 minutes to 1.0 unit of penicillin per ml.,

and the penicillin is then removed, multiplication is prevented for (on the average) $2\frac{1}{2}$ hours.

If similar effects occur *in vivo*, at least part of the reason for the paradoxical efficiency of intermittent administration of penicillin may be accounted for, since serum concentrations of this order are easily attained after intramuscular injections of 50,000 units of penicillin.

Hemolysis of Human Red Cells by Hemologous Complement, in the Presence of Tannic Acid. JOHN L. PECK and LEWIS THOMAS (Introduced by H. W. Josephs), Baltimore, Maryland.

It has long been known that tannic acid in high dilutions renders erythrocytes susceptible to lysis by complement. A study of this phenomenon was undertaken. Human cells treated with tannic acid were lysed by complement from the same individual. The effective range of tannic acid was from 0.06 to 0.008 per cent. The optimal concentration of sodium chloride was 0.7 per cent, with 1 per cent red cell suspensions.

Studies on the factors influencing the reaction were undertaken. Sensitization occurred only after exposure of red cells to tannic acid for at least 5 minutes. If tannic acid were mixed with complement before the addition of red cells, lysis did not occur. Small amounts of protein in the red cell suspension prevented the action of tannic acid. When cells had been sensitized with tannic acid, they could be washed without losing their susceptibility to lysis by complement. Inhibition of lysis was caused by heparin and congo red. Cells sensitized with tannic acid were more susceptible to lysis by detergents than normal cells.

Using tannic acid, human complement and human red cells as the indicator system, complement fixation tests were performed with various antigen-antibody mixtures. The results were comparable to those obtained in the standard test with guinea pig complement, sheep cells and rabbit amboceptor.

Antihyaluronidase Studies in Rheumatic Fever. ROBERT W. QUINN (Introduced by J. R. Paul), New Haven, Connecticut.

The early work of Friou and Wenner on the antihyaluronidase test as a diagnostic measure of activity of infection in rheumatic fever, has been confirmed in these studies. Their method which was originally described by McClean (which is a mucin-clot prevention test) has been modified to determine the antihyaluronidase titre of sera from patients with rheumatic fever in different phases of activity, patients convalescent from beta hemolytic streptococcal infections, non-streptococcal infectious diseases, rheumatoid arthritis and normal individuals.

It was found that the mean antihyaluronidase titre of sera from patients with rheumatic fever was significantly higher than the mean titre of sera from any other group of patients or normal individuals. The most important result in this study was the finding that the mean antihyaluronidase titre of sera from patients with *active*

rheumatic fever was significantly higher than the mean titre of sera from patients in any other phase of rheumatic fever or any other group of patients studied.

The rise and fall of serum antihyaluronidase titre during the active and inactive phase of rheumatic fever is also demonstrated.

Possible mechanisms involved in this test are discussed.

Relationship of Antibody Response Following Hemolytic Streptococcus Sore Throat to Development of Nonsuppurative Complications. LOWELL A. RANTZ (by invitation) ELIZABETH RANDALL, and (by invitation) HELEN H. RANTZ, San Francisco, California.

A large number of cases of Group A hemolytic streptococcus sore throat were studied in great detail. Serial antistreptolysin "O" and antifibrinolysin determinations were made over a period of 4 or more weeks. The results of this investigation demonstrate that the magnitude or frequency of the antibody response was greater when a nonsuppurative complication was a sequel to the initial acute respiratory illness. The mean increment of antistreptolysin in uncomplicated infections was 210 units per ml. When arthritis, late fever, carditis or nonsuppurative pneumonitis supervened the mean increase in this antibody was 434 units.

Similarly, the frequency of antifibrinolysin response increased from 25.3 per cent to 45.5 per cent in the presence of these poststreptococcal disorders. The results just described are statistically highly significant.

Analysis indicates that these variations are not the result of differences between the antibody stimulating properties of the hemolytic streptococci responsible for the initial infection. It is believed that the augmented formation of antibody by human beings who develop poststreptococcal nonsuppurative disease reflects an immunological hyper-reactivity on the part of these individuals.

Data will be presented in appropriate tables and charts in support of these statements, and the significance of these observations will be discussed.

The Mechanism of Rapid Fibrinolysis in Chronic Hepatic Disease. OSCAR D. RATNOFF (Introduced by G. Canby Robinson), Baltimore, Maryland.

The frequency of rapid fibrinolysis, described by Goodpasture in patients with cirrhosis, was investigated. The lysis time of recalcified plasma clots of 25 of 28 patients with cirrhosis, and 9 of 17 patients with hepatic damage secondary to other pathology, was two days or less, but was three days or more in all of 12 patients with acute hepatitis and 6 patients with obstructive jaundice, and 164 of 175 other controls.

The mechanism controlling the rate of fibrinolysis was investigated. The rate was unrelated to spontaneous, chloroform-activated, or fibrinolysin-activated plasma proteolytic activity, or to the inhibitory activity of fresh plasma or serum against plasma proteolytic enzyme. However, the inhibitory activity of all of 38 plasma tested against plasma protease decreased, during incuba-

tion at 37° C., to a constant minimal level. The time this level was reached coincided roughly with the clot lysis time. The deterioration of inhibitory activity was independent of the presence of calcium or fibrin. Thus, the rapid fibrinolysis observed in patients with chronic liver disease seemed to depend not on the presence of more proteolytic activity in such bloods, nor on a poverty of proteolytic enzyme inhibitor, but rather on the rate of inactivation of the labile inhibitor.

The Renal Extraction of Mannitol and Para-aminohippurate Compared to their Excretions in Normotensive and Hypertensive Subjects. FRANÇOIS C. REUBI (Introduced by Carl V. Moore), St. Louis, Missouri.

A comparison was made between the clearances of para-aminohippurate and mannitol and the simultaneous renal extraction of these substances before and after the injection of epinephrine and histamine in human subjects. The right renal vein was catheterized and blood samples obtained simultaneously from the femoral artery or antecubital vein and the renal vein. The apparent clearances, as measured by the formulae $C_M = \frac{U_M V}{P_M}$ and $C_M =$

$E_M \frac{C_{PAH}}{E_{PAH}}$ were compared. Considerable discrepancies were found. In one normotensive subject the clearance calculated from the extraction was as much as 54 per cent higher than that calculated from the urinary excretion. This was true to a lesser degree in one hypertensive subject with good renal function. One subject, with a possible hypernephroma and only one kidney, showed no discrepancy. Two hypertensive subjects, with a reduced apparent renal blood flow, showed the reverse: The clearance, calculated from the extraction, was as much as 31 per cent lower than that calculated from the urinary excretion. When histamine or epinephrine was injected subcutaneously, these discrepancies were reversed in all cases only to return 30 to 40 minutes later. The effect of histamine on renal blood flow, as measured by the clearance technique, was found in five hypertensive and five normotensive subjects to act somewhat similarly to that of epinephrine. Since $\frac{U_M}{P_M - R_M}$ does not equal

$\frac{U_{PAH}}{P_{PAH} - R_{PAH}}$, and since this discrepancy can be altered by the injection of these vasoactive drugs, it is possible that under some conditions mannitol may be metabolized or reabsorbed through lymphatic vessels, and under other conditions para-aminohippurate may be lost in the same manner. Renal arterio-venous by-passes do not account for these differences.

The Thyroid Inhibiting Properties of Tetrabromthyronine. (By invitation) CHARLES E. RICHARDS, (by invitation) ROSCOE O. BRADY, (by invitation) OLIVE JONESON, (by invitation) DOUGLAS S. RIGGS, and RULON W. RAWSON, Boston, Massachusetts.

The thyroid inhibiting properties of tetrabromthyronine have been evaluated by observing the effects of this agent

on the development of goiters in rats receiving thiouracil. These properties have been compared with the antigoirotropic effects of thyroxine.

It has been observed that tetrabromthyronine, when administered in a daily dose of three hundred micrograms, has an antigoirotropic effect comparable to that of thyroxine administered in a daily dose of twenty micrograms. Tetrabromthyronine in part prevented the loss of thyroid iodine, though to a lesser degree than did thyroxine.

These thyroid inhibiting properties of tetrabromthyronine were observed even though the blood protein bound iodines fell to levels comparable to those found in rats being treated with thiouracil alone. In the rats receiving thiouracil and thyroxine there was a significant increase in the blood protein bound iodine levels.

The pituitaries of animals treated with these agents have been assayed for thyrotrophic hormone by injecting suspensions of pooled pituitaries into cockerels and examining the thyroids microhistometrically. The pituitaries of rats treated with thiouracil alone were found to possess no demonstrable thyrotrophic activity. The coadministration of tetrabromthyronine or of thyroxine with thiouracil prevented any loss of thyrotrophic activity from the pituitaries.

Differentiation of Distribution- from Diffusion-Impairment in Pulmonary Emphysema and Fibrosis. R. L. RILEY and J. H. McCLEMENT (Introduced by D. W. Richards, Jr.), New York, New York.

Recently developed methods permit the estimation of the oxygen partial pressure gradient between alveolar air and arterial blood. The factors contributing to the alveolar-arterial pO_2 gradient are distribution, diffusion and venous admixture. "Distribution" refers to variations in alveolar pO_2 in different parts of the lung, hence to the ratio of alveolar ventilation to alveolar perfusion. "Diffusion" refers to the resistance of the alveolo-capillary membrane to the passage of oxygen, hence to both permeability of the tissue-fluid barrier and total area of the blood-gas interface. "Venous admixture" is a relatively insignificant factor in the absence of congenital anomalies. It is possible to differentiate impaired diffusion from impaired distribution by estimating the alveolar-arterial pO_2 gradient at high and low levels of oxygenation. Owing to the characteristics of oxyhemoglobin dissociation the distribution factor is preponderant at or near full arterial oxyhemoglobin saturation and the diffusion factor is preponderant at approximately 70 per cent. Findings in a patient with a type of fibrosis causing impaired alveolo-capillary diffusion are contrasted with those of an emphysematous patient in whom alveolar ventilation and alveolar perfusion are poorly correlated.

Studies on the Role of Histamine in Hypersensitivity to Cold. BRAM ROSE, Montreal, Canada.

Studies on the histamine content of the blood and plasma were made on eight patients with hypersensitivity to cold, following the administration of histamine and immersion of the hands and fore-arms in ice-water, both

before and after the previous administration of antihistamine compounds. In four of these patients, symptoms were not reproduced, nor was the blood histamine altered. In the remaining four, exposure to cold resulted in marked swelling of the immersed parts. In three of these, the symptoms appeared to be due to local histamine release, since marked increases of the total blood and plasma histamine occurred, and coincided with the peak of the drop in blood pressure, and increase in the pulse rate. Furthermore, the symptoms could be inhibited by the previous administration of an antihistamine compound. In the fourth patient, in addition to the swelling, there was a profound drop in the blood pressure, a marked bradycardia and syncope. The blood histamine was unchanged, and antihistamine compounds were without effect. It was concluded that histamine was not a factor in this case. These results will be discussed in relation to the histamine theory of allergy, and the effect of antihistamine compounds on the liberation and action of histamine.

Inactivation of Viruses by Secretions of the Respiratory Tract. HARRY M. ROSE and (by invitation) ELEANORA M. PRINCE, New York, New York.

The sputums of patients suffering from a number of diseases affecting the respiratory tract have been found to contain variable amounts of a substance which will inhibit the agglutination of erythrocytes by influenza virus. Sputum and sputum extracts containing a high titer of this inhibitory substance have neutralized up to 1000 minimal lethal doses of influenza virus in mice, and have also neutralized from 100 to 1000 minimal infectious doses of herpes and vaccinia viruses in chick embryos. Agglutinin inhibition tests with sputum and serum from the same patient indicate that the titer of circulating influenza antibody is not related to the concentration of inhibitor in the sputum.

Measurable amounts of the inhibitory substance have not been found in fresh or autolyzed suspensions of human liver, kidney, spleen, pancreas, salivary gland and voluntary muscle. Partially purified material has been obtained from sputum by extraction with chloroform, followed by fractional precipitation with alcohol and acetone.

The nature and significance of the virus inhibitor are unknown, but its presence in secretions of the respiratory tract suggests that it may function as a direct mechanism of defense against certain viral agents.

Variation Occurring in Group A Streptococci During Human Infections. SIDNEY ROTHBARD and ROBERT F. WATSON, New York, New York.

A study of the variation in group A streptococci which occurred during the natural course of infection in man was made. From 50 patients with 52 infections, 234 strains of recognized serological types, isolated at weekly intervals, were tested for their capacity to resist the bacteriostatic action of normal human blood and to synthesize the type-specific M protein. In 42 per cent of the infections, strains isolated in the convalescent and

carrier stages showed an increasing susceptibility to bacteriostasis correlated with a progressive loss of M substance; whereas, in the remaining 58 per cent resistance to bacteriostasis and the capacity to produce M protein were maintained throughout the observation period.

In 3 different infections, strains completely lost their capacity to synthesize the M protein and concomitantly became highly susceptible to bacteriostasis. Spontaneous reversion did not occur, but serial mouse passage re-established these functions. These degraded variants had the same T antigen as their respective original strains, which is further evidence the variants developed in the host from the initially isolated strains.

Studies were made to correlate this phenomenon with (a) the appearance of type-specific bacteriostatic antibodies in the patients' sera, (b) the serological type of streptococcus, (c) the production of streptococcal proteinase, (d) the therapeutic administration of sulfadiazine, and (e) the development of purulent complications or rheumatic fever. The possible relationship of these observations to the problem of the "dangerous carrier" of hemolytic streptococci is also discussed.

Excretion Rhythms of Water and Electrolytes in the Nephrotic Syndrome. DAVID A. RYTAND and (by invitation) J. M. CRISMON, San Francisco, California.

In a girl four years of age, the nephrotic syndrome began abruptly during a serum-sickness-like reaction to bee-sting. It was possible to examine individually some 85 consecutively voided specimens of urine (only a few were lost) through three spontaneous cycles of exacerbation and remission within a short time. Determinations included specific gravity, pH, and rates of excretion of water, sodium, potassium, chloride, and protein.

In general, a rise of urinary pH was the earliest indication of an approaching remission. This was soon followed by water diuresis, which in turn was succeeded by simultaneous increases of sodium and chloride excretion, including both concentration and rate. Urinary potassium concentration tended to be high when that of sodium was low, and vice versa. During the remissions, there was a diurnal cycle in excretion of water, sodium, and chloride in that each was excreted more rapidly early in the morning. Similar cycles have been reported by others in normal individuals.

These findings, together with those of others, suggest that chronological differences in behavior of water and electrolytes may be characteristic both of formation and disappearance of edema.

Polymyxin: Experimental and Clinical Investigations. EMANUEL B. SCHOENBACH, (by invitation) MORTON S. BRYER, (by invitation) ELEANOR A. BLISS and (by invitation) EARL OTT, Baltimore, Maryland.

"Polymyxin" is an antibiotic substance derived from cultures of *B. polymyxa* and described by P. G. Stansley, R. G. Shephard and H. J. White. It is active only against Gram-negative organisms. It is probably a basic polypeptide and appears to be similar if not identical

with "Aerosporin," an antibiotic recently described by G. C. Ainsworth, A. M. Brown and G. Brownlee in England, which was obtained from cultures of *B. aerosporus*. *In vitro*, polymyxin is active against a wide range of Gram-negative organisms and, in many instances, 0.15-0.3 micrograms per cubic centimeter has been bactericidal. The L.D. 50 of this antibiotic following injection of mice via the subcutaneous route is 0.3 gram per kilogram. Dogs tolerate 15 milligrams per kilogram administered as a single intravenous dose and 10.0 milligrams per kilogram injected intramuscularly twice daily for seven days. When 1.25 milligrams per kilogram was injected intravenously into a rabbit polymyxin was detected at two but not at three hours in the blood.

Polymyxin has not been detected in the spinal fluid after intramuscular administration. It is excreted slowly in the urine in which, 24 hours after its administration, bactericidal concentrations of the drug are noted. Polymyxin is very effective (much more so than streptomycin) against experimental infections with *Kl. pneumoniae* Type A and *H. influenzae* Type b. It also is of interest that to date, despite repeated attempts, it has not been possible to produce resistance to polymyxin *in vitro*.

Polymyxin in total daily dosage up to 5 milligrams per kilogram, given in divided doses at intervals of 3 hours, has been used for therapy of patients ill with infections due to *Ps. aeruginosa*, *Kl. pneumoniae*, and *Br. abortus*. The results thus far have been promising.

Pressor Substances in Extracts of Hypertensive Blood.

HENRY A. SCHROEDER (by invitation) MELVIN L. GOLDMAN and (by invitation) NORMAN S. OLSEN, St. Louis, Missouri.

Alcoholic extracts of hypertensive and normotensive arterial blood were prepared, concentrated, extracted with petroleum ether, and purified by subsequent alcoholic extraction. They were further purified by adsorption on anionic and cationic exchange resins and by the formation of picrates. These extracts were tested for pressor activity in the whole anesthetized rat. Their adsorption spectra and their ability to potentiate the topical action of epinephrine on the rat's mesoappendix were also determined. The color formed by Richter's method for amine picrates was also measured. Those patients (15) exhibiting arterial hypertension with renal disease, either primary or secondary but without nitrogen retention, were found to contain in their blood substances which gave a prolonged pressor response in the rat. In extracts made from the blood in all but three of fifteen exhibiting "neurogenic hypertension" no such response was found. The extract of the blood of one normotensive subject out of fifteen gave the response. The picrate color was found to be usually increased in the blood of hypertensive subjects, the average being three times as much as was found in most normotensive subjects. The extract from hypertensive subjects almost uniformly potentiated the topical action of epinephrine on the rat's mesoappendix, while that from normotensive subjects usually did not. It can be concluded that pressor sub-

stances can be demonstrated in the blood of some hypertensive individuals.

Nitrogen Balance Studies on the Kempner Rice Diet.

WILLIAM B. SCHWARTZ and JEROME K. MERLIS (Introduced by Maurice B. Strauss), Framingham, Massachusetts.

Kempner's report that nitrogen balance is achieved on a rice, fruit, and fruit juice diet with a nitrogen intake estimated to be 3.2 gm. per day is not in accord with other data on minimal nitrogen requirements. In Kempner's studies no analyses of food or fecal nitrogen were reported. Nitrogen balance studies were carried out on six normotensive subjects who adhered strictly to the Kempner rice regime for eight days following a four-day period on a nitrogen depletion diet. One patient with severe hypertension was studied for a 90-day period on the rice regime alone.

The actual nitrogen content of the rice regime, determined by macro-Kjeldahl analysis, was 2.63 gm. rather than Kempner's estimated 3.2 gm. On the eighth day of the rice diet the average total N excretion (urinary plus fecal) was 5.85 gm. in the normotensive subjects, giving a negative N balance of 3.22 gm. per day. The curve of urinary nitrogen excretion indicated that basal values had been closely approximated. On the 90th day the hypertensive patient had a total N excretion of 5.30 gm. per day with a negative balance of 2.67 gm. per day.

The Metabolism of Silver. (By invitation) K. G. SCOTT and J. G. HAMILTON, San Francisco, California.

A study of the metabolism of carrier-free radioactive silver has been made in the rat at intervals from 1 to 64 days. The total quantity of silver, containing the radioactive silver, administered to each animal was less than .001 microgram. Parenteral administration was followed by rapid elimination, most of which took place by way of the liver, the excreted silver appearing in the feces. Absorption by way of the digestive tract was noted to be less than .1 per cent of the administered dose. It has been shown that the normal route of excretion takes place by way of the bile, since ligation of the bile duct reduces the fecal excretion by a factor of more than 10. When the carrier-free radio-silver was diluted by the addition of inert silver, in the range of .1 to 1 milligram of silver administered to each animal, the distribution in the tissue and excreta became very different, there being a ten to one hundred-fold increase at the earlier time intervals of radio-silver in organs such as liver, kidney, spleen, skin, bone, and muscle; and a marked decrease of its rate of excretion. It appeared that the deposition in these tissues was proportional to the total amount of silver administered. A marked degree of decrease in the excretion of silver by way of the liver took place following 3 hours of light chloroform anesthesia. This effect was found to be transient with return to the normal rate of excretion within six days after the administration of the anesthetic. It is suggested that these results point the way for the de-

development of a liver function test. It was found that *in vivo* and *in vitro*, most of the carrier-free silver in the blood is bound in a globulin fraction and is not dialyzable to any extent through cellophane.

The Synergistic Action of Streptomycin and Sulfadiazine in the Therapy of Experimental Brucella Infection in the Developing Chick Embryo. (By invitation) JAMES M. SHAFFER and WESLEY W. SPINK, Minneapolis, Minnesota.

Investigations in this clinic have revealed that combined therapy with streptomycin and sulfadiazine is the most effective treatment available to date in both bacteriologically proved human brucellosis and experimental *Brucella* infection in the chick embryo. This report is concerned with the mechanism whereby such a therapeutic combination is more effective than when either agent is used alone. It has been shown that the superiority of the combined therapy is due to a typical synergistic action of the two therapeutic agents. This synergism is present in experimental infections established with *Br. abortus*, *Br. suis* or with *Br. melitensis*.

The synergistic action of streptomycin and sulfadiazine has been demonstrated by treating infected chick embryos 24 hours after infection with small doses of streptomycin or sodium sulfadiazine alone, and with the two combined. Typical experimental results against *Br. Abortus* show that 80 micrograms of streptomycin or 0.12 milligram of sodium sulfadiazine will not eliminate *Brucella* from any of the embryos, but when these drugs are given together in these doses *Brucella* are eradicated from 40 per cent of the infected-treated chick embryos. By doubling the above doses, the combined therapy produced 75 per cent negative cultures as compared to 30 per cent for sodium sulfadiazine alone, and 10 per cent for streptomycin alone. Such experimental results provide supporting evidence for the use of streptomycin and sulfadiazine in human brucellosis.

The Low Potassium Syndrome in Chronic Nephritis. (By invitation) SOL SHERRY, LUDWIG W. EICHNA and DAVID P. EARLE, JR., New York, New York.

A patient with persistent hypokalemia (1.5-2.5 meq./L.), hypochloremia (85-94 meq./L.), and hypotension (80/50) exhibited transient muscular weakness, abnormal cardiac rhythms, and electrocardiographic abnormalities. Evidence is presented that the clinical syndrome was due to a faulty mechanism of the renal tubules for the handling of potassium, secondary to renal disease. Ammonium chloride administration revealed little defect in ammonia or titratable acidity production, but a low potassium diet promptly led to a negative potassium balance, weakness and cardiac abnormalities. Normal serum potassium levels could not be attained by the prolonged daily administration of 25 grams of KCl.

Potassium depletion affected the heart in two ways, (a) by increasing the vagal effect and (b) by flattening and broadening the T wave of the electrocardiogram. These effects could be separated by atropine.

The height of the T wave of the electrocardiogram could be correlated with the serum potassium level in acute experiments after potassium administration. However, in the post absorptive state the correlation was poor. A single dose of potassium produced a transient rise in serum potassium level, whereas a slow and progressive improvement in muscle strength began several hours later. The evidence after potassium administration suggested a rapid distribution of potassium into certain spaces, followed by a slower redistribution into others.

The Effect of "Tracer Doses" of Radioactive Iodine on the Function of Chick Thyroids. BENGT N. SKANSE, PRISCILLA MERRILL and ROBLEY D. EVANS (Introduced by Oliver Cope), Boston, Massachusetts.

We have studied the effect of radioactive iodine (I^{131}) on the thyroid's growth, iodine content and response to thyrotrophic hormone in cockerels. These effects were studied sixteen and twenty-four days after administering the I^{131} .

Thyroids which collected 0.1 microcurie were not altered in growth or iodine concentration. Growth of the thyroids which collected 1 and 5 microcuries was significantly inhibited. Iodine concentration of the 1 microcurie group was not altered; however, there was a significant decrease in concentration of thyroid iodine in the 5 microcurie group.

All irradiated animals responded to thyrotrophic hormone as measured by increase in thyroid weight and loss of iodine sixteen days after administering I^{131} . However, at the twenty-four-day interval there was demonstrated a dissociation in response to thyrotrophic hormone between the 1 and 5 microcurie groups. In the first group a loss of iodine was observed but no increase in thyroid weight. In the latter group there was no effect on either iodine loss or thyroid weight.

We have demonstrated that so-called tracer doses of I^{131} may alter normal functions of the thyroid and that these functions vary in their sensitivity to irradiation with this isotope.

Cardiovascular Dynamics in Experimental Embolism of Restricted Portions of the Lungs. JOHN R. SMITH and (by invitation) MASAKI HARA, St. Louis, Missouri.

Studies of experimental pulmonary embolism indicate that small single or multiple emboli may produce marked elevation of pulmonary arterial tension and death, with intense dilatation of the right cardiac chambers. Excluding massive pulmonary arterial obstruction, these dynamic changes from smaller emboli suggest "reflex" pulmonary vascular spasm.

In open-chest dog preparations, carotid arterial and pulmonary arterial pressures were recorded. Flexible rubber catheters were introduced into the main pulmonary artery and directed into a selected lobar arterial branch. The injection of small quantities of barium sulfate or potato starch suspensions into the selected lobar artery provoked a striking rise of pulmonary arterial tension, a fall of systemic pressure, and death, with evidence of

overwhelming right heart failure. Microscopic study indicates embolization of the pulmonary capillaries. The experiments suggest that pulmonary vascular reflex spasm may be induced by capillary embolization of highly restricted lung portions.

In other experiments, catheters were placed in selected lobar arteries, but the main artery to the corresponding lung was ligated. Embolization with barium or starch was then ineffective. Subsequent removal of the ligature resulted promptly in death with right heart failure. The experiments suggest that reflex pulmonary spasm may be abolished by interruption of nerve tracts contained in the pulmonary arterial walls.

Acceleration of Flow in the Veins of Human Limbs by the Local Application of Pressure. (By invitation) JOSEPH R. STANTON, (by invitation) EDWARD D. FREIS and ROBERT W. WILKINS, Boston, Massachusetts.

The velocity of blood flow in the veins of the limbs was determined fluoroscopically, or with serial roentgenograms, by timing the progression of 4 cc. of 35 per cent diodrast injected in a distal vein. Following control observations in which the pattern as well as the velocity of venous flow was noted, the extremity was evenly pressurized by the inflation of cuffs smoothly applied to the limb prior to the experiment. At pressures of 12-40 mm. Hg the velocity of flow was increased above that of the control observations. Likewise, in three experiments with both lower limbs studied simultaneously, an increase in velocity of venous flow occurred in the pressurized as compared with the unpressurized extremity. The distribution of the diodrast in the venous bed was not significantly altered by the application of pressure although the diameter of the individual veins was decreased.

These observations, which seem pertinent to the clinical problem of phlebothrombosis in both medical and surgical patients, suggest that the velocity of venous flow in the limbs may be increased by mild local compression. The apparent explanation for the observed acceleration is that such compression decreases the total cross-sectional area of the venous bed proportionately more than it reduces the volume of blood flow as previously reported from this laboratory.

Effect of Sodium Chloride Depletion on Blood Pressure and Tetraethyl Ammonium Chloride Response in Hypertension. WILLIAM W. STEAD and MORTON F. REISER (Introduced by M. A. Blankenhorn), Cincinnati, Ohio.

Temporary autonomic ganglion block can be produced by tetraethyl ammonium chloride (TEAC), thus leaving a "floor" pressure which is probably maintained by intrinsic vascular and humoral mechanisms.

Blood pressure and TEAC responses were studied in 11 patients with severe progressive hypertension during: (1) control periods, (2) salt deprivation (0.25 gm. dietary sodium per day), and (3) re-salting. In patients without severe renal insufficiency the sodium deprivation was supplemented by mercurhydrin thrice weekly. In all

patients de-salting produced a comparable degree of dehydration and fall of serum sodium, but the changes in blood pressure and TEAC response fell into two distinct groups:

(1) Gradual fall in resting pressure and even greater fall in the TEAC "floor" during sodium deprivation and return to control values during re-salting. This response occurred in ten experiments in five patients. All but one had large initial TEAC response.

(2) No essential change in either resting or "floor" pressure. This occurred in eight experiments in six patients, generally with renal impairment, only one patient having fairly adequate renal function at the outset. None had large initial TEAC response.

The data suggest variations in the contribution of neural and humoral factors in different patients. When the humoral component was predominant de-salting produced little effect, but it was effective when neural factors were in the foreground.

Potassium Deficiency and the Role of the Kidney in its Production. (By invitation) R. TARAIL and J. R. ELKINTON, New Haven, Connecticut.

Potassium was administered without cardiotoxic effects in daily doses of 1.43 to 3.65 milliequivalents per kilogram to six adult patients maintained on parenteral fluids. Five of the patients were losing gastro-intestinal fluid, and the sixth had had a cerebral vascular accident. Two normal subjects were given 3.42 and 4.37 milliequivalents of potassium per kilogram, as controls. The exchanges of electrolytes and nitrogen were measured.

In 4 of the patients the concentrations of potassium in serum were abnormally low. All of the patients retained administered potassium in the cellular phase in "excess" of nitrogen, in amounts varying from 1.20 to 4.61 milliequivalents per kilogram. Only one patient received potassium long enough to show that the maximum degree of retention had been reached. The 2 normal subjects retained only 0.29 and -0.14 milliequivalents per kilogram.

During periods of low potassium intake more potassium was lost in urine than in gastro-intestinal fluid. In 4 patients the quantity in urine was greater than during periods when the intakes were high and the concentrations in serum were normal. The minimum amounts in urine in 3 of the patients deprived of exogenous potassium and maintained in nitrogen equilibrium, were 28, 27, and 6 milliequivalents per day. The renal tubules did not reabsorb potassium completely under these conditions of maximum need for conservation of the ion. The data indicate the primary role of the kidney in the production of potassium deficiency in these patients.

Studies on the Mechanism of the Schwartzman Phenomenon. LEWIS THOMAS and CHANDLER A. STETSON, Jr. (Introduced by Harold E. Harrison), Baltimore, Maryland.

Approximately two hours after intravenous injection of Schwartzman's meningococcal filtrate in rabbits, exten-

sive hemorrhagic reactions in the abdominal skin resembling Shwartzman reactions could be induced by the intradermal injection of cysteine or BAL (2,3, dimer-captopropanol). These substances did not cause hemorrhages in normal animals. Hemorrhages were not produced by Na ascorbate, glutathione, or by any of a large number of unrelated compounds. Hemorrhages with the thiol compounds could only be elicited between two and five hours after intravenous injection of bacterial filtrate.

Similar but more extensive hemorrhages occurred when papain was injected intradermally two hours after intravenous bacterial filtrate. In control animals, papain caused much smaller areas of localized necrosis, or produced no reaction.

When skin was prepared for the Shwartzman reaction by intradermal bacterial filtrate, a single application of bromobenzene to the area at any time during the next 20 hours caused complete inhibition of the reaction. Other lipid solvents had a similar effect.

It is postulated that the Shwartzman phenomenon may be due to the action on blood vessels of a tissue protease, activated by sulfhydryl groups. One phase of the reaction may consist of the withdrawal of protease-inhibitor from the involved tissue.

Elaboration of Hyaluronidase by Pneumococci Isolated from Bacteremic Pneumococcic Pneumonia Patients.

ROBERT T. THOMPSON and FRANCES E. MOSES (Introduced by Morton Hamburger, Jr.), Cincinnati, Ohio.

Previously reported rises of antihyaluronidase titer in the sera of patients with bacteremic pneumococcic pneumonia indicate that pneumococcus hyaluronidase was elaborated early in these infections. These findings pose the question: Will pathogenic pneumococci which elaborate hyaluronidase retain this property during culture in ordinary artificial medium? Pneumococci from nine bacteremic pneumonia patients were passed through 0.05 per cent glucose broth every eighteen to twenty-four hours, and then were tested at intervals for the ability to elaborate hyaluronidase by subculture into 0.20 per cent hyaluronic acid broth.

Five of the nine pneumococci tested elaborated hyaluronidase at the first subculture into hyaluronic acid broth, as follows: Four pneumococci were first tested eighteen hours after culture from the patients, and three of these elaborated hyaluronidase; three were first tested thirty-six hours after culture from the patients, and two of these elaborated hyaluronidase; two were first tested forty-eight hours after culture from the patients, and neither elaborated hyaluronidase.

Four of the same five pneumococci which elaborated hyaluronidase on first subculture into hyaluronic acid broth failed to do so on second subculture at intervals of two days, two days, three days, and four days respectively after culture of the pneumococci from the patients. The other pneumococcus which elaborated hyaluronidase on first subculture was not subsequently tested.

These findings indicate that pathogenic pneumococci which elaborate hyaluronidase lose this property in ordi-

nary broth medium approximately forty-eight hours after removal from the pneumonia patient.

Urine "Corticosteroids" in Toxemia and Hypertension.

LOUIS TOBIAN, JR. (Introduced by Tinsley R. Harrison), Dallas, Texas.

Urinary "corticosteroids" were extracted with ethyl acetate and determined by the Loewenstein method which is not necessarily specific. All measurements in pregnant patients were made in the 31-40 weeks.

The results obtained were as follows:

1. In normal late pregnancy, "corticosteroids" were twice the nonpregnant value.

2. Women with twins excreted approximately 40 per cent more than comparable single-fetus women.

3. Pregnant women with excessive edema, with or without toxemia, excreted 50 per cent more "corticosteroid" than pregnant women with minimal or no edema.

4. Mild preeclampsics with minimal or no edema excreted no more "corticosteroid" than nontoxemics.

5. Edematous mild preeclampsics excreted as much "corticosteroid" as equally edematous women with more severe preeclampsia.

6. Nonpregnant patients with essential hypertension, one diabetic, and one nephrotic, all had essentially normal "corticosteroid" excretion.

The results obtained would suggest that increased excretion of "corticosteroids" is correlated, not with hypertension but with pregnancy, and more especially, with edema developing during pregnancy. Although this conclusion cannot be considered established until a larger series of patients has been studied, the findings may afford a partial explanation for the similarity of preeclampsia to desoxycorticosterone intoxication.

Acetylcholine and Neuronal Activity in Craniocerebral Trauma. (By invitation) DONALD B. TOWER and DONALD McEACHERN, Montreal, Canada.

During studies on the presence of acetylcholine and cholinesterases in cerebrospinal fluids of over 100 neurological patients, interesting observations have been made on a group with craniocerebral trauma. Detailed methods and results are given elsewhere. Patients fall into 3 groups: (a) Epileptics—"normal" cholinesterases, acetylcholine present. (b) Craniocerebral trauma patients—cholinesterases abnormal, acetylcholine present in varying amounts. (c) Normal individuals and patients with various diseases—cholinesterases "normal," acetylcholine absent.

Only the second group of 14 craniocerebral trauma cases is considered in detail here. Serial observations have been possible in some cases. Low CSF cholinesterase activity and reversal of normal cholinesterase ratios characterize the group. In severe cases acetylcholine is present in the cerebrospinal fluid in large amounts. Recovery is associated with reversal of the above changes. In 3 cases correlation is illustrated between cholinesterase pattern, acetylcholine level, EEG and the clinical state of

the patients. Reference is also made to 6 psychiatric patients undergoing electroshock therapy (a form of craniocerebral trauma) who evidenced similar changes.

Bornstein showed in animals the presence of acetylcholine and abnormalities of the EEG following artificially induced craniocerebral trauma. Our studies in man indicate a correlation between chemical and electrographic findings and the clinical state of the patient and thus contribute to the understanding of brain injury.

Zinc and Carbonic Anhydrase Content of Red Cells in Normals and in Pernicious Anemia. BERT L. VALLEE (Introduced by John G. Gibson, 2nd), Boston, Massachusetts.

Zinc is a component of the enzyme carbonic anhydrase. The zinc content (measured by dithizone), and the carbonic anhydrase activity (measured by the velocity of CO_2 evolution), of normal packed red cells, have a constant relationship. Normal unit values for packed red cells range from 11 to 19 gamma of zinc, averaging 14.7; and from 2.6 to 5.1 E units of carbonic anhydrase, averaging 3.9 per cc.

In the anemias due to iron and dietary deficiency, infection and uremia, unit values of both zinc and carbonic anhydrase are within normal limits. There is a marked increase in both the metal and enzyme in untreated pernicious anemia. Values obtained in 8 patients ranged from 19.0 to 30.0 gamma of zinc, averaging 24; and from 5.9 to 12.7 E units of carbonic anhydrase, averaging 8.0. The ratio of the components, therefore, was comparable to that found in normal red cells. Under successful liver therapy both components return to within normal limits in about 60 days; the relative proportion of metal and enzyme is identical to that of normal cells. Under maintenance therapy values remain normal.

In secondary anemias, zinc and carbonic anhydrase decrease on a slope parallel to that of the drop in hemoglobin. In contrast, in pernicious anemia, the increase of zinc and carbonic anhydrase is inversely proportional to the fall in hemoglobin. This may indicate that the hemoglobin and carbonic anhydrase systems are structurally discrete although functionally related.

Susceptibility of Red Cells and Serum Factor in the Mechanism of Hemolysis in Paroxysmal Nocturnal Hemoglobinuria. PHILIP F. WAGLEY and MAURICE D. HICKEY (Introduced by William B. Castle), Boston, Massachusetts.

In patients with paroxysmal nocturnal hemoglobinuria, the number of red cells that are susceptible to hemolysis may be determined by the repeated incubation for 45 minutes of the cells in samples of fresh human serum at pH 6.4 until no more hemolysis occurs. In one patient the presence of hemoglobinuria correlated with the number of such susceptible red cells. The susceptible cells varied from 3 to 33 per cent of the total red cell population. Hemolysis of such cells is enhanced *in vitro* when the physiological pH range is decreased from 7.4 to 7.22.

The factor in human serum required for the hemolysis of red cells from patients with paroxysmal nocturnal hemoglobinuria is inactivated by procedures known to inactivate the complement required for hemolysis of sheep cells sensitized by amboceptor. However, no complement fixation is observed in the hemolytic mechanism in paroxysmal nocturnal hemoglobinuria and the hemolytic activity of human serum is rapidly diminished with only slight changes in complement activity when fresh human serum is diluted with serum previously heated to 57° C. for 30 minutes. Following the restoration of pH to approximately 6.3 of sera previously incubated at 37° C. at a pH of 5.1, 6.2, 7.4, complement activity for sensitized sheep cells was still demonstrable. However, for the hemolytic system of red cells from a patient with paroxysmal nocturnal hemoglobinuria, hemolysis was irreversibly inactivated by the incubation of serum at a pH of 5.1 and 9.2; hemolysis was not restored by the addition of guinea pig complement to the system adjusted to pH 6.4. The factor in human serum required for hemolysis in paroxysmal nocturnal hemoglobinuria is not identical with the complement required for hemolysis of sensitized sheep cells.

The Effects of Histamine Administered Intravenously on the Peripheral Circulation in Man. (By invitation)

KHALIL G. WAKIM, (by invitation) GUSTAVUS A. PETERS, (by invitation) JEAN C. TERRIER and BAYARD T. HORTON, Rochester, Minnesota.

The effects of the continuous intravenous administration of histamine diphosphate on skin temperature, blood pressure, heart rate and blood flow were studied among patients who were receiving the drug therapeutically. The drug was administered to each patient in a solution of 1:250,000 in saline at successive rates of 0.004, 0.008, 0.016 and 0.024 mg. of histamine per minute, respectively. The duration of infusion at each rate was twenty minutes. Control values for skin temperatures, heart rate, blood pressure and blood flow were established before the infusion of histamine was started, and the observations were repeated at regular intervals thereafter for each of the periods of infusion at each of the four infusion rates and for five to fifteen minutes after the infusion was stopped. The blood flow in all four extremities was determined by means of the plethysmograph with a compensating spirometer recorder. The cutaneous temperatures were recorded galvanometrically by means of skin thermocouples applied to the forehead, to the skin over the right and left deltoid muscles, and over the right and left quadriceps femoris muscles.

Histamine produced a cutaneous vasodilatation which appeared first over the face and neck of the patient and gradually extended downward over the upper extremities and thorax, reaching the lower extremities only toward the end when the higher rates of infusion were used. There was a definite increase in skin temperature and in heart rate, and a slight decrease in blood pressure.

The blood flow in the four extremities gradually increased until at the highest rate of infusion of 0.024 mg.

histamine per minute, the average increase in blood flow in the 12 subjects was 182 per cent in the forearms and 45 per cent in the legs, over the control values. However, five minutes after the infusion of histamine was stopped, the blood flow averaged only +46 per cent in the forearms and +27 per cent in the legs. The changes in skin temperature, blood flow, heart rate and blood pressure gradually subsided, and the values returned toward the control level shortly after cessation of the infusion of histamine.

The Balance of Sodium and Potassium in Repair Solutions. WILLIAM MCLEAN WALLACE (Introduced by James L. Gamble), Boston, Massachusetts.

The administration of sodium has long been known to depress the potassium balance of the body and vice versa. The loss of intracellular potassium in the fasting-thirsting state has been shown to have significance with respect to the efficacies of repair solutions. The quantity of potassium which can be provided for replacement of this loss is limited by the concentration which is considered safe as regards cardiac function and the maximal practicable volume. The question then presents: to what extent should the provision of sodium for repair of deficit be limited in order to obtain the most efficient utilization of potassium? As a part of studies of infants receiving treatment for severe diarrhea and acidosis, attempt has been made to answer this question by daily measurements of balance for the individual electrolytes over periods of treatment in which differing quantities of sodium were provided along with 3 m.mol of potassium per kg. of body weight in a volume of 200 cc. per kg.; taken as limits in terms of safety and practicabilities. The data describe the desirability of reduction of the quantity of sodium ordinarily used in fluid therapy to an extent which permits an approximately parallel progress of replenishment of sodium and potassium deficits.

Influenza Virus Associated with Bacterial Pneumonia.

THOMAS G. WARD, ELIZABETH STARBUCK MAXWELL and THOMAS E. VAN METRE, JR. (Introduced by Thomas B. Turner), Baltimore, Maryland.

It is well known that an increase in pneumonia cases accompanies epidemics of clinical influenza. Evidence presented herein suggests that influenza virus is one of the causative agents in cases of pneumonia previously considered primarily bacterial in origin. Sputum was obtained from 69 cases of bacterial pneumonia and studied for influenza virus by the chick embryo technique. Of 33 cases occurring during non influenza periods one yielded an influenza "B" virus. Of 36 cases occurring during the period when influenza "A" was prevalent in Baltimore 13 yielded influenza virus which were serologically similar to influenza "A" strains isolated from clinical cases of influenza occurring at the same time.

Acute and convalescent blood specimens were secured on 53 of these same cases and a third specimen was obtained about five months later on 25. Four cases showed

evidence of positive hemagglutination-inhibition antibody response but failed to yield virus.

Thus, a total of 17 of 36 cases (47 per cent) of bacterial pneumonia occurring during an influenza "A" epidemic gave evidence, by virus isolation or serologic techniques, of the presence of influenza virus associated with bacterial pneumonia.

The significance of these findings in the pathogenesis of bacterial pneumonia will be discussed.

Studies on the Action of the Heart by Means of a Cine-radiographic Technique. J. V. WARREN, (by invitation) H. S. WEENS and (by invitation) D. F. JAMES, Atlanta, Georgia.

Radiographic contrast visualization of the heart is a means of extending our knowledge of cardiac activity in health and disease. The value of serial angiocardiograms is limited since they depict only isolated phases of the cardiac cycle. More complete information may be obtained utilizing slow motion cineradiography.

Small mongrel dogs were used for the present experiments. Under pentobarbital anesthesia contrast medium (diodrast or thorotrast) was injected rapidly into the superior vena cava through an intravenous catheter. Motion pictures were made of the image produced on a high speed fluoroscopic screen by x-rays generated at 90 kilovolts and 100-150 milliamperes. Utilizing cameras equipped with large aperture lenses it was possible to record 30 to 40 frames per cardiac cycle on either 16 or 35 mm. green sensitive film. Although projection of the motion pictures permits an overall slow motion demonstration of cardiac activity, more detailed analysis must be based upon the study of individual frames.

Twenty observations have been made on normal anesthetized dogs. Despite variations in the position of the animal and the medium injected, the results were essentially the same in all. The superior vena cava and right atrium were opacified almost immediately following injection of the contrast medium. In proper succession visualization of all heart chambers, the major pulmonary vessels, and the aorta was obtained. In many instances the position and motion of the atrioventricular valves could be determined. Of particular interest was the incomplete emptying of all heart chambers which was noted in every instance. The amount of residual blood in the ventricles, as well as in the atria, during systole was more than anticipated. This may well be of importance in explaining the ability of the heart to undergo the extremely rapid changes in cardiac output known to occur. Increases or decreases in stroke volume may be the result of alterations in the amount of residual blood.

Metabolic Studies on Protein Depleted Patients Receiving a Large Part of their Nitrogen Intake from Human Serum Albumin Administered Intravenously. CHRISTINE WATERHOUSE and JACOB HOLLER (Introduced by Samuel H. Bassett), Rochester, New York.

The availability of purified albumin preparations has led to studies on their utilization in man. The conversion of

albumin to tissue protein, variations dependent on routes of administration, and the induced changes in renal function have been demonstrated by other investigators. That important variations in individual response can occur is illustrated by the following experiments.

Each of three subjects received a daily dose of 60 gm. of concentrated Na free human albumin for 10 or more days. Balances of N, Ca, P, and K were made during control, albumin, and post-albumin periods. Concomitant observations of serum protein fractions, plasma volume and renal function were made.

Subject 1, a man convalescent from rheumatic fever and without evidence of impaired renal function, developed an intense proteinuria by virtue of which he was in negative nitrogen balance on the 10th day of therapy. In subject 2, an emaciated woman with fever of unknown etiology and probable increase in capillary permeability, about 40 per cent of the injected albumin appeared to leak into the extracellular fluid, while 50 per cent was catabolized. The remainder was excreted in the urine or converted to tissue protein. There was marked retention of water with edema, hydrothorax and pericardial effusion. Subject 3, a young woman, was in fair health except for moderate undernutrition. Her response in most respects followed along the lines reported by other investigators, i.e., an immediate, marked retention of the albumin with subsequent slow conversion of 50 per cent of the quantity injected to tissue protein; catabolism of 40 per cent; excretion of 1.6 per cent in the urine with the rest chiefly in the plasma. After the eleventh day of therapy signs of cardio-respiratory embarrassment were noted.

Proteinuria appeared to bear no relation to kidney damage as judged by measurements of glomerular filtration and renal blood flow. It is suggested that this phenomenon may be expected to occur whenever large doses of albumin are administered for a sufficient period of time.

Coronary Flow in Experimental Auricular and Ventricular Tachycardias. RENÉ WÉGRIA and (by invitation) RICHARD P. KEATING, New York, New York.

Coronary flow and arterial blood pressure were recorded in anesthetized dogs during experimental auricular and ventricular tachycardias. A rate of stimulation equal to that of the spontaneous rhythm was first employed, then it was progressively increased.

In auricular tachycardia of rates approximating the spontaneous rate, no change occurred. With rates higher than the spontaneous rate, a transient drop in flow and pressure was followed by their return to control levels and, when the rate was not excessive, the flow reached a level above its control. As the rate of tachycardia increased, flow and pressure decreased more markedly and even remained below control levels. When tachycardia produced initially a drop of flow and pressure, its termination was followed by an increase of flow and pressure. The higher the rate of tachycardia, the greater was the increase in flow and pressure upon the termination of tachycardia.

In ventricular tachycardia, essentially similar phenomena occurred but the decrease in flow was more marked and lasted longer.

The mechanisms of the phenomena observed are discussed.

Some Effects upon Nitrogen Balance of the Independent Variation of Protein and Calories in Man. SIDNEY C. WERNER, New York, New York.

In most nutritional studies involving reduction of calories or of protein, both variables have been altered concurrently. The effect of change of either of these factors independently has needed detailing. Such a study has been carried out. The results appear to have significance in respect to outlining intravenous as well as oral feeding programs and in respect to the interpretation of the mechanism behind the loss of nitrogen from the body following trauma or disease. The present study has been divided into four parts:

1. A reduction of calories at constant protein intake. This results in negative nitrogen balance of a degree to equal that resulting from most traumatic reactions.

2. The restitution of nitrogen balance at low caloric intakes. This can be done by increasing the protein nitrogen intake despite a simultaneous reduction in carbohydrate intake necessitated thereby to keep calories constant.

3. A study of the effect of equicaloric fat versus carbohydrate reduction at a constant nitrogen intake level. There is a greater negative nitrogen balance with carbohydrate reduction as opposed to fat reduction reaffirming the greater nitrogen sparing effect per unit of carbohydrate over fat even at relatively high intake levels of both.

4. A study of the effect of protein reduction after high protein intakes, with constant caloric intake. A sharp negative nitrogen balance occurs even at high caloric levels, the duration of which may exceed a week.

From these data it is concluded that much of the loss of nitrogen after injury may result from caloric reduction and from an adaptation to a high protein nitrogen turnover level. This high level may be the result of absorption of protein nitrogen from injured tissues. These data also offer possible suggestions in respect to the goal of treatment for pre- and post-operative nitrogen feeding.

An Analysis of the Unresponsiveness to Mercurial Diuretics Observed in Certain Patients with Severe Chronic Congestive Failure. RAYMOND E. WESTON and DORIS J. W. ESCHER (Introduced by Louis Leiter), New York, New York.

In a series of cardiac patients who no longer gave satisfactory responses to organic mercurial diuretics, renal clearances of sodium, chloride, mannitol (GFR), and PAH (R.P.F.) were determined before and after administration of mercuzanthin, and again after the rates of sodium and chloride filtration were increased by either the rapid intravenous administration of aminophyllin (0.48–0.72 grams) or the continuous infusion of 4.5 per

cent NaCl (at times, plus molar Na lactate). A similar procedure was carried out on one non-edematous, hypertensive patient in whom a very low GFR was produced by the Kempner rice diet.

In nearly all cases, the very low control water and salt excretion rates were not significantly affected by the mercuzanthin alone. However, after giving the mercuzanthin, if the filtration of sodium and chloride was increased by injection of aminophyllin or concentrated salt solution, there was a marked rise in urinary water and salt output, in some instances to values approximating those observed in cardinals responsive to mercurials. It is concluded that the previous failure of these patients to respond to mercurial diuretics was due not to the usually postulated renal tubular resistance to mercury, but rather to the marked decrease in sodium and chloride filtered. The significance of these data with respect to the relationship between impaired renal hemodynamics and salt retention in chronic congestive failure will be discussed.

Familial Incidence of Neurocirculatory Asthenia ("Anxiety Neurosis," "Effort Syndrome"). (By invitation) EDWIN O. WHEELER, (by invitation) PAUL D. WHITE, (by invitation) ELEANOR REED and MANDEL E. COHEN, Boston, Massachusetts.

Family histories from patients with neurocirculatory asthenia (N.C.A.) have suggested that it is a familial disorder. To investigate further the familial incidence of N.C.A. the sons and daughters of patients with N.C.A. were examined to determine whether or not they had N.C.A.

The family study was based on 50 patients in whom the diagnosis of N.C.A. had been made 20 years before its verification in this study. 22 families had 45 children over 18 years of age. The 37 available children from 18 families were examined. For control data, the prevalence of N.C.A. was determined from 5 groups comprising 234 individuals, 129 women and 105 men. The diagnosis of N.C.A. was based on its characteristic symptoms.

The prevalence of N.C.A. in the sons and daughters of parents with N.C.A. was 48.6 per cent. In contrast, the prevalence was only 5.6 per cent in the control groups. This difference is highly significant statistically, the significance ratio being 5.1 (Odds: 1.7×10^6 to 1). These data do not reveal whether the disorder is hereditary or on an acquired household basis.

It is concluded that the prevalence of N.C.A. in the sons and daughters of patients with N.C.A. is significantly higher than in the general population.

A Source of Error in Metabolic Rate Determinations Resulting in Falsely Low as well as Falsely High Values. HAROLD N. WILLARD and GEORGE A. WOLF, JR. (Introduced by David P. Barr), New York, New York.

Apparent metabolic rates varying from more than minus one hundred to plus one hundred as measured by the Benedict-Roth closed spirometer apparatus could be produced voluntarily by a trained subject. These tests

were classified as satisfactory by experienced technicians after inspection of the patient and the tracing.

Patients and normal controls were studied using pneumographs around the abdomen and chest, the tilt table and its effect on the diaphragm, and the estimation of complementary air before and after metabolic rate determinations.

Progressive change in the chest volume occurring during the determination of the metabolic rate, rather than change in the oxygen consumption, was shown to cause the marked discrepancy between the apparent and the true metabolic rate.

The relation of these observations to the value and interpretation of clinical determinations of basal metabolic rate will be discussed.

Reciprocal Relationships of Radioiodotherapy and Thyroid Function. ROBERT H. WILLIAMS and (by invitation) HERBERT JAFFE, (by invitation) WALTER F. ROGERS, JR., (by invitation) BEVERLY T. TOWERY, and (by invitation) RENE TAGNON, Boston, Massachusetts.

Studies conducted with 175 individuals and several hundred rats, considered in conjunction with previous reports, indicate that the turnover of radioiodine in the thyroid gland is influenced by many factors, e.g., (a) the size and structure of the thyroid, (b) the amount and duration of thyrotoxicosis, (c) severe trauma, infection, emotional reactions, starvation, and extreme changes in temperature, (d) the amount of iodine ingested before and after the radioiodine, and (e) the amount, duration, and interval of cessation of treatment with antithyroid drugs. Some of the details of these studies will be presented.

Therapeutic doses of radioiodine have been given to 105 patients, consisting of 101 unselected subjects with thyrotoxicosis, 2 with non-toxic nodular goiter, and 2 with malignant adenoma. All patients were given I^{131} except for 3 who received I^{130} . Each of these 3 subjects were administered 25 or 30 millicuries and has been free of thyrotoxicosis for more than one year. The remaining 98 patients with hyperthyroidism were given an average of approximately 8.5 millicuries of I^{131} in from 1 to 6 doses. In 18 cases therapy has been too recent to afford much evaluation. Seventy-six patients have been in a euthyroid state for from 3 to 12 months, while 4 are myxedematous. The individuals with myxedema had received a total of from 4 to 7 millicuries, but each had been treated with one of the thiouracils for more than one year.

Some of the patients experienced an exacerbation in the thyrotoxicosis during the 2 weeks following therapy. Treatment with one of the thiouracils before and with iodide after the radioiodine helped to prevent these reactions. The largest quantity of protein-bound radioiodine in the serum was found approximately 7 days later, the concentrations gradually decreasing during the next 5 weeks. Patients given potassium iodide for 5 days after the I^{131} obtained maximal concentrations of protein-bound radioiodine approximately 2 weeks after the I^{131} .

It required about 6 weeks or longer to obtain the maxi-

mum response in the thyrotoxicity. In most of the cases treated the goiter disappeared and no evidence of damage to other tissues has been found. A moderate reduction in the size of the thyroid resulted in the 2 patients with non-toxic goiters. All palpable thyroid tissue in the cases with malignant adenoma disappeared.

The plan of therapy with I^{131} that we now use for most of the thyrotoxic patients is as follows: (a) one of the thiouracils, and no iodide, is given for about 5 weeks, (b) 4 days after cessation of this therapy an average of 100 microcuries of I^{131} , with 0.5 mg. of potassium iodide, is given orally, (c) beginning one day later 3 drops of a saturated solution of potassium iodide is given twice daily for 5 days, and (d) another dose of I^{131} is given approximately 6 to 8 weeks later if hyperthyroidism exists.

Effects of Attitude and Conditioning on Action of Chemical Agents in Human Subjects. The Pharmacology of Placebos. STEWART WOLF, New York, New York.

Fifty experimental observations were carried out on twelve human subjects in an attempt to obtain quantitative data concerning the "placebo" action of chemical agents. The meaning of "placebo" is extended to apply to any action of a substance other than that attributable to its pharmacologic properties.

The chief subject was Tom, a man with large gastric fistula, whose stomach lining was accessible to view. In this individual the effects of drugs on the stomach were measured by kymographic recordings of motor activity, analysis of gastric juice and color photography of the gastric mucosa as well as by direct visualization.

It was found that pharmacologically "inert" substances such as distilled water and lactose exert a readily measurable "placebo" action. For example, under experimental circumstances after suitable conditioning with prostigmine it was repeatedly observed over a period of four weeks that the administration of a lactose capsule induced hyperaemia, hyperacidity and hypermotility in the stomach and hypermotility in the colon of greater degree and duration than was observed following the prostigmine itself. Furthermore, measurable "placebo" effects could be demonstrated in the case of pharmacologically active agents such as atropine and benedryl. At times the "placebo" effect of a drug cancelled out or appreciably outweighed its usual pharmacologic action. For example, at a time of anxiety concerning the experimental procedure, the action of atropine occasioned, instead of its usual inhibiting effect, hyperaemia, hyperacidity and hypermotility in the stomach.

Simultaneous Studies of Intraradial and Intrafemoral Arterial Pressure Before and After Corrective Surgery for Coarctation of the Aorta. E. H. WOOD, G. E. BROWN, JR., H. B. BURCHELL and O. T. CLAGETT (introduced by C. F. Code), Rochester, Minnesota.

Continuous and simultaneous recordings of intraradial and intrafemoral arterial pressures, venous pressure, electrocardiographic data and respiration have been obtained from 6 normal subjects and from 27 patients who had

coarctation of the aorta, with these persons at rest in the horizontal position and during various cardiovascular tests. When the patients were in the horizontal position: (1) systolic and, in most instances, diastolic, pressure in the radial artery was elevated above the range of values obtained in the normal subjects, (2) systolic pressure in the femoral artery was reduced or within the range of values obtained in normal persons, while diastolic pressure was, in most instances, above the normal range, (3) the femoral/radial systolic pressure ratio and the femoral/radial pulse pressure ratio were below the range of the normals, (4) the onset of the femoral pulse wave was nearly always delayed beyond the onset of the radial pulse wave and (5) the period of time elapsing between the onset and the attainment of the peak in the femoral pulse wave was, with one exception, beyond the range of the comparable period of time as obtained among normal subjects.

Postoperative studies have been carried out among 12 patients. The data demonstrate that the cardiovascular dynamics of patients who have coarctation of the aorta may be altered toward the normal state, and the data constitute an objective measure of the degree of this alteration.

Observations on Hemolytic Reactions Produced in Dogs by Transfusion of Incompatible Dog Blood. LAWRENCE E. YOUNG and (by invitation) CHARLES L. YUILE, (by invitation) DONALD M. ERVIN and (by invitation) EDWARD VON HASSELN, Rochester, New York.

Dogs systematically transfused with dog erythrocytes containing a factor lacking in their own red cells developed isohemagglutinins and hemolysins that exhibited characteristics of immune antibodies. Dogs thus immunized were transfused under controlled conditions with incompatible whole blood in order to provide opportunities for studying the pathological physiology of hemolytic reactions.

In each instance base-line observations were made and followed by closely spaced post-transfusion measurements of hematologic, immunologic and chemical alterations. These measurements included total white cell and differential counts, determinations of osmotic and mechanical fragility of red cells, coagulation time, prothrombin concentration, serum antibody and complement titers, serum and urinary potassium, plasma hemoglobin, urea nitrogen, bilirubin and electrophoretic pattern, clearances of hemoglobin, mannitol and creatinine and estimations of effective renal plasma flow.

The transfused cells were tagged with Fe^{59} which made it possible to determine accurately the rate of destruction of donated corpuscles and the rate of excretion of the hemoglobin thus liberated. The disappearance of transfused cells was also followed by the technique of differential agglutination (Ashby). Studies are in progress on the effects of dehydration and acidosis and the administration of various therapeutic agents that may aid in correcting the sequelae of rapid red cell destruction.

This type of controlled transfusion experiment in dogs provides a new approach to the investigation of both immediate and delayed effects of the combination of hemoglobinemia and antigen-antibody reactions under conditions closely simulating those encountered clinically.

The Role of Muscle Mass and of Renal Reabsorption in Creatinuria in Man. (By invitation) K. L. ZIERLER, (by invitation) J. W. MAGLADERY, (by invitation) B. P. FOLK and J. L. LILIENTHAL, JR., Baltimore, Maryland.

Consideration of the main pathways concerned in the normal metabolism of creatine suggests that creatinuria may be the result of accelerated synthesis of creatine, extrusion of intracellular creatine, inadequate disposition of creatine, or diminished renal tubular reabsorption of creatine. The role of two of these factors was evaluated in human subjects.

(1) Determinations of nitrogen balance, guanidoacetic acid excretion, serum concentrations of creatine and of creatinine, and urinary excretion of creatine and creatinine, spontaneously and under creatine loads, in obsolete anterior poliomyelitis, were interpreted as indicating that reduction in muscle mass alone is an adequate cause of creatinuria.

(2) Simultaneous measurements of creatine clearance and of glomerular filtration rates revealed that the ability of the renal tubule to reabsorb creatine was diminished (a) during the administration of thyroid substance in hypothyroidism, (b) during prolonged administration of desoxycorticosterone acetate, and (c) during the puerperium.

It is concluded that the creatinuria may be independent of altered muscle metabolism. Furthermore, it would appear, from the examples discovered in the case of creatine, that speculation concerning extra-renal mechanisms may not be justified by simple measurement of urinary

excretion unless appropriate determinations of renal function have been undertaken.

The Flow Through the Coronary Bed in Normal and Abnormal Human Hearts by the Method of Kerosene Perfusion. P. M. ZOLL and D. T. DRESDALE (Introduced by H. L. Blumgart), Boston, Massachusetts.

The rate of inflow into each coronary artery and the partition of outflow via the right and left chambers were measured. The hearts were then studied by the Schlesinger technique.

Under standard conditions and constant pressure the rate of inflow is an index of the resistance of the entire coronary bed.

1. In the presence of coronary occlusions, the rate of inflow per gram weight of heart was reduced in contrast to normal hearts; the rate per gram of tissue did not increase with cardiac hypertrophy.

2. The rate of inflow into one coronary artery increased up to 15 per cent when perfusion through the other artery was stopped. This increment was not affected by the presence of occlusions.

3. When one coronary artery was perfused, the outflow from the uninjected coronary artery varied from 1-12 per cent of the inflow. Transmitted pressures up to 18 mm. of mercury were observed in the latter artery.

4. The aortic outflow in normal hearts was less than 5 per cent of the total outflow, possibly representing small left-sided Thebesian flow; in hearts with occlusions, it was usually increased, from 14 to 37 per cent, and radio-paque mass injected into the coronary arteries was found occasionally in the left ventricle.

5. These observations indicate that narrowing or occlusion of a coronary artery leads to increased luminal-coronary communications as well as the previously described interarterial collateral channels.

THE EFFECT OF ANEMIA AND POLYCYTHEMIA ON DIGITAL INTRAVASCULAR BLOOD VISCOSITY¹

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Viscosity is a fundamental property of matter, and its mathematical analysis has challenged numerous physicists, including Newton (1) and Einstein (2). It is not our purpose to review the mathematical formulas evolved for the determination of viscosity, but rather to attempt to apply existing principles as simply as possible to a study of the human circulation.

The physical forces involved in any study of intravascular viscosity must always begin with the law elaborated by Poiseuille (3). Most viscometers measure the flow of fluid at a given pressure through a tube of a definite length and diameter, and are based on Poiseuille's law. Viscometers dependent on other principles (4, 5) are less analogous to conditions which prevail in any study of intravascular viscosity. The Ostwald (6) and the Hess (7) type of viscometer have hence been most commonly employed for studying the viscosity of blood *in vitro*. In the former, the velocity of the blood flowing through the tube is low. Hess (8) showed that when the velocity is increased the viscosity decreases, but that within certain optimum ranges of velocity there is little if any change in the viscosity. These optimum velocities are of the order usually found under physiological conditions. At higher velocities flow becomes turbulent, and the relations between pressure, flow, tube diameter and velocity again change (9). Since physiological velocities and those employed in viscometers are such that flow is streamlined, turbulence may be disregarded.

It has also been shown that the viscosity of a fluid decreases as its temperature rises (10). This is believed to be due in part to changes in the volume of the fluid with increasing temperature (10) and in part to other factors (11). In addition, very viscous fluids do not flow at all below a certain minimum pressure (12). This intercept of the pressure-flow ratio line on the pres-

sure coördinate is referred to by Bingham and Roepke (13) as the "yield value." These authors therefore suggest that only the fluidity, which is the reciprocal of the viscosity, be used as a measure of the flow characteristics of a fluid, and that all fluidities be corrected for the temperature factor, arbitrarily designating 20° C. as the point of reference for comparative measurements. Since all viscosities or fluidities are thus relative to water at 20° C., the terms "relative" or "specific" fluidity or viscosity have been used (5) to indicate this.

In studies of the effect of tube diameter on the viscosity of blood Fåhræus (14), and later Suter (15) found that below a certain critical diameter the viscosity of blood did not follow the law of Poiseuille, but decreased with decreasing diameters. Fåhræus explained this by pointing out that in capillary tubes the cells are crowded toward the center of the stream because of their greater weight leaving a comparatively larger peripheral zone of plasma where the greater frictional stresses occur. Since it was thus clear that the viscosity of the blood could change with variations in velocity on the one hand, and tube diameter on the other, the terms "apparent" (12) or "apparent specific" (5) viscosity or fluidity were used to indicate that such measurements were not absolute but were only applicable to the particular system in which the viscosity was studied.

In the more recent modifications of the Poiseuille formula (9) the variables are pressure, volume flow, density, length of tube, radius of tube, mean velocity and viscosity. If the length and cross-sectional area of a viscometer remain constant and the mean velocity is within such a range as to produce a constant effect, these factors taken together—namely, length, cross-sectional area, and velocity—may be considered a single constant. The pressure-flow relationship at a given temperature and density will hence be linear in a viscometer in which all other factors

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become a constant (12). If the only factor changed in such a system is the viscosity of the flowing liquid then the change in frictional resistance to flow with varying viscosity becomes expressed by a change in the slope of the pressure-flow line (12). If the intercept of the pressure-flow line or "yield value" were zero, the relationship $\frac{P}{F} K$ would accurately reflect changes in viscosity, where P = the pressure in mm. of Hg,
 F = the flow in gm. per unit of
 tissue per minute

and K = a constant.

When the line intercepts the pressure coördinate above zero, as it does with viscous fluids, $\frac{P - P_1}{F} K$ now represents the viscosity where P_1 is the intercept or "yield value."

In 1933 Whittaker and Winton (12) realized that the variable factors in viscosity measurements made it impossible to draw accurate conclusions as to the behavior of fluids in blood vessels from *in vitro* viscometer studies. They therefore used the hind-limb of a dog to study pressure-flow relationships directly and to determine the effect of changes in viscosity on these relationships. Maximal vasodilatation and stabilization of vasomotor tone were effected by the addition of chloral hydrate to the perfusing blood. In their favorable experiments the pressure-flow relationship was linear. They established the magnitude of the intercept which varied from 7 mm. for plasma to 27 mm. for blood containing 83% by volume of cells. They found intravascular blood viscosity to be lower than viscometer measurements, as might be expected from Fåhræus' observations (14). They also found that the viscosity increased very gradually with increasing erythrocyte concentrations at anemic levels and more precipitously at polycythemic levels.

In 1938 (16) a method was developed for measuring blood flow and blood pressure in the fingertip after stabilization of the circulation by indirect heating. The normal range of values was narrower than for any other such clinical method for measuring blood flow. This method was adapted in 1942 (17) to the measurement of peripheral resistance, a crude value representing merely the ratio of pressure to flow multiplied by a constant. Since the velocity factor can be assumed to be

relatively constant under these conditions it follows that the pressure-flow ratio expresses changes in the viscosity if the cross-sectional area factor remains unchanged. The cross-sectional area was kept constant by releasing sympathetic tone by indirect heating (16) in each observation. Since the pressure-flow relationship is linear under these conditions, changes in viscosity could be determined by a change in the ratio of pressure to flow, however the individual values for each of these were altered. There was, however, no feasible method for the determination of the magnitude of the pressure intercept in the human digital circulation. For that reason Whittaker and Winton's intercepts (12) were substituted in the hope that the error of such a step would be sufficiently small to enable pressure-flow relationships to be considered linear.

Digital arterial diastolic and systolic pressures were determined by a Gaertner capsule (16, 18). The arithmetic mean pressure was considered satisfactory despite the minimal error entailed. The intercept for blood of such erythrocyte concentration as prevailed was subtracted from this mean pressure. Venous pressure was considered included in the intercept value.

Flow was measured calorimetrically (19), using the formula

$$F = \frac{(\Delta t_1 + \Delta t_2)(m + e)}{sa(t_s - t_a)}, \text{ in which}$$

F = the flow in gm. per sq. cm. per min. in the fingertip,
 Δt_1 = the rise in temperature per min. in the calorimeter,
 Δt_2 = the fall in temperature per min. after the fingertip is removed,
 m = the volume in cc. of the water in the calorimeter,
 e = the hydrothermic equivalent of the calorimeter and the fingertip,
 s = the specific heat of the blood,
 a = the area of immersed fingertip in sq. cm.,
 t_s = the mouth temperature in degrees C.,
 and t_a = the average calorimeter temperature.

The specific heat was originally taken to be 0.9 according to Stewart (20). It was apparent, however, that this constant might change with varying erythrocyte concentrations and that some effort should be made to determine the exact specific heat of plasma and erythrocytes. Direct calorimetric studies of blood (21) revealed the specific heat of plasma to be 0.94, and of cells 0.77. It was also possible to predict the specific heat of any mixture of cells and plasma. Using these values for specific heat, the flow levels were corrected accordingly. Normal digital blood flow now became 0.24 to 0.35 gm. per sq. cm. per min. Using the arbitrary constant of 0.3, the normal

resistance measured in the manner described above now became 52 to 86 units. F is expressed in grams because the numerator of the equation represents gram-calories. To convert to cc. it is necessary to divide by the specific gravity of the blood.

We studied four patients with polycythemia treated by repeated phlebotomy and also two anemic patients. In one patient with aplastic anemia the erythrocyte concentration was brought up to a normal level by transfusions, and in the other with pernicious anemia, by folic acid. Table I lists results obtained in the patients with polycythemia and Table II, in those with anemia. In

the final column the changes in resistance with varying erythrocyte concentrations are listed using the resistance of each patient at normal erythrocyte levels (considered unity) as the point of reference. These ratios therefore represent changes in viscosity relative to that of normal blood. Changes in erythrocyte concentration are represented by changes in hematocrit. In some instances, changes in hemoglobin or erythrocyte count per cu. mm. were converted into hematocrit values by the use of Wintrobe's factors (22). It is clear that no one measurement of erythrocyte concentration is entirely satisfactory in this re-

TABLE I
Polycythemia

Name	Age and sex	Diagnosis and therapy	Date	Hematocrit	Digital blood flow	Digital arterial blood pressure	Intercept	Digital vascular resistance	Ratio of abnormal to normal viscosity
					<i>gm. per sq. cm. per min.</i>	<i>mm. Hg</i>	<i>mm. Hg</i>		
L. L.	52 M	Primary polycythemia treated by repeated phlebotomy	3/14/47	61	0.20	88 — 72	19	92 units	1.39
			3/17/47	62	0.19	94 — 60	19	92	1.39
			3/24/47	51	0.21	81 — 55	17	73	1.11
			4/18/47	43	0.24	80 — 54	14	66	1.00
J. L.	60 M	Primary polycythemia treated by repeated phlebotomy	9/17/41	65	0.14	88 — 66	20	122	1.36
			10/6/41	49	0.19	94 — 64	15	101	1.12
			10/15/41	42	0.21	90 — 64	14	90	1.00
L. C.	36 M	Primary polycythemia treated by repeated phlebotomy	7/2/42	73	0.19	120 — 100	23	137	1.60
			7/6/42	66	0.23	120 — 76	20	102	1.26
			7/10/42	59	0.24	118 — 94	19	100	1.34
			7/21/42	53	0.21	100 — 74	17	100	1.24

TABLE I—Continued

Name	Age and sex	Diagnosis and therapy	Date	Hematocrit	Digital blood flow	Digital arterial blood pressure	Intercept	Digital vascular resistance	Ratio of abnormal to normal viscosity
					<i>gm. per sq. cm. per min.</i>	<i>mm. Hg</i>	<i>mm. Hg</i>		
			7/25/42	47	0.23	$\frac{104}{78}$	15	98	1.21
			7/30/42	45	0.27	$\frac{112}{84}$	14	93	1.15
			8/4/42	43	0.28	$\frac{106}{74}$	14	81	1.00
A. M.	54 M	Primary polycythemia treated by repeated phlebotomy	3/29/47	61	0.20	$\frac{85}{50}$	19	74	1.35
			4/11/47	59	0.22	$\frac{84}{52}$	19	67	1.22
			4/4/47	56	0.22	$\frac{74}{50}$	18	60	1.09
			4/16/47	54	0.24	$\frac{82}{54}$	17	64	1.16
			4/23/47	53	0.21	$\frac{75}{48}$	17	63	1.15
			4/28/47	50	0.22	$\frac{72}{48}$	16	60	1.09
			4/30/47	47	0.23	$\frac{76}{50}$	15	62	1.13
			5/5/47	42	0.25	$\frac{70}{50}$	14	55	1.00

spect. It is well known that in polycythemia the mean corpuscular volume may be small and that it also varies considerably in anemia. Mean corpuscular hemoglobin concentration varies even more (22). Furthermore, there are variations in the hematocrit of capillary, venous and arterial blood (23) and corrections to be made for plasma "trapped" in the cell mass (24). It was our purpose, however, to determine if any change at all could be demonstrated and to approximate the magnitude of the change in the living human subject. It can be seen from Table II that the decrease in viscosity in anemia was small. In polycythemia, the viscosity increased at first slowly

with increasing cell concentrations and then precipitously. The increased viscosity produced a decrease in flow in polycythemia and little change in blood pressure, whereas in anemia the relationship between flow and pressure with increasing cell concentrations was variable. In Figure 1 the ratio of normal to abnormal viscosity is plotted against erythrocyte concentration. Whittaker and Winton's (12) curve is drawn in. The observed points correspond well with this curve. One might by extrapolation and assumptions as to the normal blood viscosity convert these figures into fluidities in rhes, or into dynes per sq. cm., but it was felt that this was not justified by the data.

TABLE II
Anemia

Name	Age and sex	Diagnosis and therapy	Date	Hemato-crit	Digital blood flow <i>gm. per sq. cm. per min.</i>	Digital arterial blood pressure <i>mm. Hg</i>	Intercept <i>mm. Hg</i>	Digital vascular resistance	Ratio of abnormal to normal viscosity	
R. S.	31 F	Aplastic anemia treated by transfusions	4/14/42	17	0.36	$\frac{88}{60}$	10	52 units	.80	$\frac{1}{1.25}$
			4/15/42	19	0.36	$\frac{86}{65}$	10	54	.82	$\frac{1}{1.22}$
			4/18/42	22	0.36	$\frac{98}{68}$	11	60	.91	$\frac{1}{1.10}$
			4/24/42	22	0.32	$\frac{90}{62}$	11	61	.92	$\frac{1}{1.09}$
			4/27/42	26	0.32	$\frac{94}{68}$	11	65	.98	$\frac{1}{1.02}$
			4/30/42	31	0.30	$\frac{86}{60}$	12	61	.92	$\frac{1}{1.09}$
			5/6/42	35	0.31	$\frac{92}{62}$	13	62	.94	$\frac{1}{1.06}$
			5/8/42	38	0.30	$\frac{92}{58}$	13	62	.94	$\frac{1}{1.06}$
			5/11/42	42	0.29	$\frac{92}{64}$	14	66	1.00	$\frac{1}{1.00}$
						$\frac{94}{56}$	11	68	.81	$\frac{1}{1.23}$
A. M.	54 M	Pernicious anemia treated with folic acid	1/9/47	21	0.28	$\frac{94}{56}$	11	68	.81	$\frac{1}{1.23}$
			1/14/47	25	0.26	$\frac{94}{48}$	11	70	.83	$\frac{1}{1.21}$
			1/21/47	30	0.29	$\frac{104}{58}$	12	71	.85	$\frac{1}{1.18}$
			1/28/47	34	0.27	$\frac{106}{64}$	13	80	.95	$\frac{1}{1.05}$
			2/4/47	36	0.28	$\frac{116}{65}$	13	82	.98	$\frac{1}{1.02}$
			3/15/47	38	0.31	$\frac{120}{80}$	14	83	.99	$\frac{1}{1.01}$
			3/28/47	42	0.32	$\frac{122}{84}$	14	84	1.00	$\frac{1}{1.00}$

* The ratios are rearranged in this column to correspond with the graph of Whittaker and Winton (12) in order that the points observed in Figure 1 may be compared with the curve obtained by these authors.

It should be pointed out that the viscosity of the blood has been found to increase with vasoconstriction (25). In flow through capillaries (26) moreover, or through the renal vessels where diffusion is extensive, the changes in viscosity become sufficiently complex to defy mathematical analysis (27). It has already been pointed out that viscosity is influenced by the caliber of the perfused blood vessels, so that the peripheral intravascular blood viscosity may be different from the viscosity of blood flowing through larger vessels or through the heart. The correspondence of the values presented here with those of Whittaker and Winton (12) strongly suggests that we were both measuring corresponding changes in blood viscosity as it affected resistance to flow at given pressures through arteriovenous anastomoses and widely dilated capillaries of living tissues, uninflu-

enced by variations in the caliber of the perfused vessels.

SUMMARY AND CONCLUSIONS

Studies on intravascular blood viscosity in the digital circulation confirm similar studies in animals. They indicate that decreases in erythrocyte concentrations such as are found in anemia decrease the blood viscosity moderately. Increases in erythrocyte concentrations such as are found in polycythemia increase the blood viscosity moderately at lower levels and more steeply at higher levels. At those extremes of anemia and polycythemia which were observed the blood viscosity was found to be 80% of normal at a hematocrit level of 17 and 169% of normal at a hematocrit level of 73.

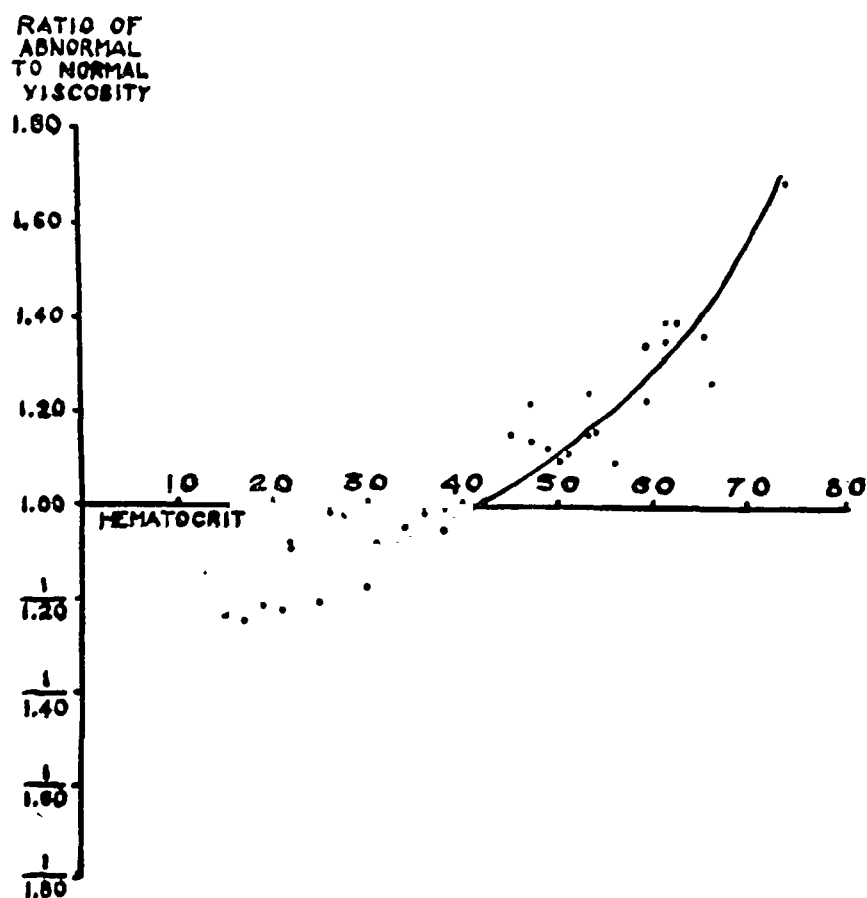


FIG. 1. THE BLACK DOTS REPRESENT OBSERVED CHANGES IN HUMAN INTRAVASCULAR VISCOSITY WITH VARYING ERYTHROCYTE CONCENTRATIONS IN SIX PATIENTS

The curve is drawn in from Whittaker and Winton (12) making allowances for a slightly higher normal hematocrit of dogs and for the fact that flow is expressed volumetrically rather than gravimetrically.

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HOMOLOGOUS AND HETEROLOGOUS ANTIBODY RESPONSE OF INFANTS AND CHILDREN TO MULTIPLE INJECTIONS OF A SINGLE STRAIN OF INFLUENZA VIRUS^{1, 2}

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It has been shown that repeated inoculation of immune animals (1, 2) does not ordinarily result in significant increases in antibody titer to the homologous strain but that when immunity declines a second inoculation may serve to increase the serum antibody titers well above the level reached after a primary infection. In contrast to normal experimental animals, the common experience with man has been that the antibody response to influenza virus reaches its height after a single inoculation of virus and that repeated inoculations within short intervals do not result in higher levels. Moreover, the accumulated data give little evidence of an exaggerated antibody response to a second stimulus administered after an interval of several months when antibody titers may be declining. It has been repeatedly suggested that the difference observed in the behavior of man and experimental animals is related to the fact that the former usually has already acquired antibody as a result of experience with the disease while the latter is a normal, inexperienced individual. In man the antigenic stimulus is presumed to be limited by the presence of antibody previously acquired (3, 4).

Early in the studies of the development of antibodies to different strains of influenza virus it was demonstrated in ferrets that repeated inoculations of the PR8 strain could result in the production of antibodies to a strain of swine influenza virus (5). This broadening in the antibody pattern with multiple injections was subsequently noted when rabbits, or mice were employed (5 to 8).

It was of interest, then, to consider whether infants and young children, who had been sub-

jected to little or no exposure to influenza, when given repeated injections of influenza virus vaccine would exhibit a broadening in antibody coverage against antigenically different strains even though the height of response to the homologous strain was little changed. The present report comprises results of a study undertaken to answer this question.

There was no evidence that influenza A had been prevalent in Michigan since the winter of 1943-44. Hence a group of young human individuals with average age of 3.2 years and largely without antibody to selected strains of influenza virus was chosen for the study.

MATERIALS AND METHODS

Subjects

The children were from the Sarah Fisher Home of the House of Providence in Farmington, Michigan. Approximately 90 children were divided into three groups. Their average age was 3.2 years. The age composition of each of the three groups was essentially as follows:

Age in years	No. of children
1-1.9.....	5
2-2.9.....	7
3-3.9.....	7
4-4.9.....	9
5-5.9.....	3
Total.....	31 (average age 3.2 years)

Children with allergic tendencies were omitted from the study groups. It is worth noting that eggs represented a substantial portion of the diet and any egg sensitization probably would have become readily evident a short time after entering the home. During the course of the study an occasional child missed a scheduled dose of vaccine because of illness, discharge from institution or other reason.

There had been no previous program of vaccination with influenza virus at the home. However, concurrent with the outbreak of type B influenza in the winter of 1945-46 in this area (9, 10) there had been a sharp increase in the number of children with elevations of temperature and upper respiratory disease in the cottages

¹ This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, United States Army, Washington, D. C.

² Also aided by United States Public Health Research Grant.

where daily temperatures were recorded. There was no evidence of a rise in the rate of acute respiratory disease during the spring and summer of 1946.

Vaccine

The vaccine was freshly prepared and furnished through the courtesy of Dr. Herald C. Cox of the Lederle Laboratories. It represented allantoic fluid from embryonated eggs infected with the PR8 strain that was concentrated 20 times in a Sharples centrifuge. It contained 0.1% formalin and merthiolate in a dilution of 1:2,500. The protein content, determined by micro-Kjeldahl nitrogen, was 2.0 mg. per ml. Before being sent to this laboratory it was tested for hemagglutinating titer using the technic of Hirst and Pickels (11), and showed a titer of hemagglutinins of 2,140.³ Tests done in this laboratory with the Salk method (12) of chicken erythrocyte agglutination resulted in a titer of 10,240 for the same preparation. Using the latter method the preparation when diluted twice gave a titer of 5,120. Further dilution to 1:15 was made and this gave a titer of 640. Another portion of the same batch of vaccine was diluted 1:72. The titer of agglutinins for chicken erythrocytes was 128. In the vaccination program 0.5 ml. amounts of the above dilutions were given subcutaneously unless indicated otherwise.

Administration of vaccine

Group I was given a large dose subcutaneously every other week for five doses. Group II was given a small dose subcutaneously every other week for five doses and Group III was given the more dilute preparation every other day for five doses. The schedule for the administration of the vaccine preparations is listed in Table I.

Group I received the first dose of 0.1 ml. of the 1:2 dilution of vaccine on 8-27-46. A number of severe febrile toxic reactions occurred and the amount was subsequently reduced. On 9-11-46, 9-25-46, 10-8-46 and 10-24-46, the children in this group received 0.5 ml. of the 1:15 dilution of vaccine; a total of five doses at two-week intervals. Prior to each dose 0.02 ml. of the 1:72 dilution of vaccine was given intradermally in the left forearm. The children were then observed during the next 30-minute period for the development of local reactions at the site of the skin test. If even minimal erythema was present 0.1 ml. of the vaccine was given subcutaneously and the child observed for an additional 30-minute period. No further untoward effects were noted, and the remaining 0.4 ml. in the dose was given subcutaneously.

Group II received 0.5 ml. of the 1:72 dilution of vaccine on 8-28-46, 9-12-46, 9-26-46, 10-9-46 and 10-25-46. Skin tests, as in Group I, were also carried out.

Group III was given vaccine every other day. The first dose given on 8-29-46 consisted of 0.5 ml. of the

³ Throughout the remainder of this report the titer will be expressed as the reciprocal of the final dilution of virus or serum.

TABLE I
Vaccination and bleeding schedule for the three groups of children

Group No.	Dosage of vaccine	Dates of administration	Dates of bleeding
I. Large dose every other week—five times.	0.10 ml. of 1:2* 0.50 ml. of 1:15†	8-27-46 9-11-46 9-25-46 10-8-46 10-24-46	8-27-46 9-11-46 10-8-46 11-6-46 4-29-47
II. Small dose every other week—five times.	0.50 ml. of 1:72	8-28-46 9-12-46 9-26-46 10-9-46 10-25-46	8-28-46 9-12-46 10-9-46 11-7-46 4-30-47
III. Small dose every other day—five times.	0.30 ml. of 1:72† 0.50 ml. of 1:72†	8-29-46 8-31-46 9-2-46 9-4-46 9-6-46	8-29-46 9-21-46 5-2-47

* First dose. † Third dose. ‡ All other doses.

1:72 dilution. This amount was repeated on 8-31-46. Some reactions occurred approximately six hours after both injections. On 9-2-46 the amount of vaccine was reduced to 0.3 ml. and the eight children who had reactions after the first and/or second doses did not receive any vaccine on this date. However, all but one child received the fourth and fifth injections of 0.5 ml. on 9-4-46 and 9-6-46. After the fourth dose only one febrile reaction occurred and none after the last injection.

Serological studies

The schedule for withdrawal of blood specimens in the three groups with the exact dates is summarized in Table I. It can be seen that additional blood samples were obtained approximately six months after the last dose of vaccine. This will be presented in a later report.

The Salk method (12) of determining the agglutination-inhibiting antibodies in the collected sera was used. Four agglutinating units of antigen were mixed with the serially diluted sera. The action of the blood sera in inhibiting the agglutination of four type A strains and two swine strains was measured. The type A strains used were: PR8 (13); Weiss (14); Baum, isolated in New York in 1941; and Olson, isolated in 1943 in Dr. Eaton's laboratory in California. The two strains of swine influenza virus, 1976 and Oti, were obtained from Dr. R. E. Shope. The passage history of the strains used is as follows: PR8—seven ferret, 593 mouse, and 56 egg passages; Weiss—three ferret, 32 mouse, and 41 egg passages; Baum—nine ferret, ten mouse, and 19 egg passages; Olson—seven egg passages. It is not known how many times the swine strains had been passed through swine: 1976 had 38 mouse and 17 egg passages; Oti had 179 mouse and 39 egg passages. All strains of virus were inoculated into ten day old embryonated eggs and the allantoic fluid was removed after incubation for 48

hours at 35° C. Passages were maintained until the allantoic fluids showed consistently high agglutination titers of virus with chicken erythrocytes. Then, large pools of allantoic fluid containing virus were prepared and clarified by adsorption and elution from red cells and restored either to the original volume or concentrated ten or 20 times in salt solution (15), after which they were stored for use in the tests. Several preliminary triplicate agglutination tests were carried out with the eluted virus preparations (Table II). The average titer

TABLE II

Average titers of the different strains of virus used in agglutination-inhibition test

Virus strain	Aggl. titers*	Titer used in tests
PR8	2560	1:640
Weiss	5120	1:1280
Baum	2560	1:640
Olson	2560	1:640
Swine 1976	2560	1:640
Swine—Oti	2560	1:640

* Done in triplicate on five successive days.

of five different triplicate agglutination tests was used to determine the amount of each virus used in the agglutination-inhibition tests. Pooled erythrocytes were obtained from the same chickens for all tests. A further check on the consistency of the results was made by the use of the same standard pools of sera of high and low titer as controls in each test. All sera from an individual were tested at the same time.

RESULTS

It was first noted that the prevaccination titers of the individual sera from the three groups followed a definite age pattern when the tests against the PR8 strain were analyzed. Only one of 54 children under four years of age had a serum antibody level greater than 32 (Table III). Conversely, of 22 children, four years of age or older,

TABLE III

Prevaccination agglutination-inhibition titer against the PR8 strain of type A influenza virus in relation to age of children whose sera were tested

Age in years	Agglutination-inhibition titer		Total no. of sera
	32 or less	64 or greater	
1-1 11/12	9	0	9
2-2 11/12	21	0	21
3-3 11/12	24	1	25
4-4 11/12	15	11	26
5-6	7	1	8

12 had initial titers of 64 or greater. This would suggest that the majority of the group had had no appreciable previous experience with influenza virus. This lack of experience was more evident in the individuals under four years of age, and corresponded to the epidemiological information that influenza A had not been prevalent in this area for at least three years.

The data were then analyzed as follows: 1) modified mean antibody titers (10) for each group (Table IV); 2) charts showing the distribution of antibody titers against each test strain of virus in each group (Figures 1 to 3); 3) modified mean titers of selected individuals in each group (the basis for the selection was those individuals who had all the scheduled doses of vaccine and bleedings and whose prevaccination titers were less than 32; these latter children were thought to represent individuals with little or no experience with influenza viruses [Table V]); and 4) the mean titers in each group measured against the PR8 strain and tabulated according to age (Table VI).

TABLE IV

The modified arithmetic mean titers of the three groups expressed as the reciprocals of the highest final dilution of serum causing inhibition of agglutination

Strain	Group I (31 individuals)				Group II (28 individuals)				Group III (31 individuals)	
	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding
<i>Type A</i>										
PR8	<32	716	1126	1024	<32	128	409	338	<32	666
Weiss	32	625	1024	952	34	247	445	451	<32	742
Baum	174	1126	1536	1423	200	435	819	435	193	1188
Olson	<32	371	417	371	40	128	192	235	<32	256
<i>Swine</i>										
1976	<32	54	43	32	<32	<32	<32	<32	<32	38
Oti	<32	128	119	113	<32	41	51	51	<32	78

TABLE V

The modified arithmetic mean titers of the sera of individuals in the three groups with (a) prevaccination titer of <32, (b) all the scheduled doses of vaccine and (c) all the scheduled bleedings

Strain	Group I (19 individuals)				Group II (14 individuals)				Group III (19 individuals)	
	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding
Type A										
PR8	<32	417	835	850	<32	100	256	230	<32	512
Weiss	<32	221	579	612	<32	86	163	197	<32	256
Olson	<32	116	179	173	<32	37	64	64	<32	170

In calculating the means all sera with titers of less than 32 were assigned the value of 16. This reduced the mean prevaccination titers and masked those individuals in the groups who had high initial titers. However, these latter sera are demonstrated in the figures showing the distribution of titers in the three groups. Also there are differences in the numbers of individuals who are included in some of the tables. This is occasioned in one instance by the fact that accurate ages were not obtainable; consequently they could not be assigned properly in the tables where age is an important factor. Some sera became contaminated and could not be titrated. The prevaccination titers against the Baum strain were so high, owing to non-specific inhibition, that the results with that antigen were not included in the selected data. Such factors contribute to the slight variations in tabulated data.

The findings summarized in the tables demonstrate that repeated inoculations within limited time intervals, regardless of dosage, resulted in cumulative increases in antibody titer (Table IV and Figures 1 to 3). This fact is not in keeping with effects commonly noted in adult humans, but

approaches more that observed in experimental animals. Whether or not these differences are significant can not be definitely stated when the data are viewed only in Table IV where the mean titers of the entire groups are presented. However, as seen in Table V where the mean titers of selected groups are given, the differences follow the same trend observed in Table IV but are more pronounced. Further analysis, using age as a basis for grouping, shows that when subsequent injections of vaccine are administered homologous antibody levels in the children of Group I under four years of age continue to rise beyond the level reached after a single injection. In the children over four, in which some antibody is originally measurable, the maximal titer is observed after the first dose, strongly indicating an accelerated response. These older children were more likely to have had previous experience with influenza. In Group II, where smaller amounts of virus were given, a continued rise was noted in children of both age groups.

The distribution of antibody titers, presented in the three figures shows with each subsequent bleeding a more pronounced grouping effect. The

TABLE VI

Mean titers in the three groups measured against PR8 strain and tabulated according to age

	Group I Large dose every other week $\times 5$				Group II Small dose every other week $\times 5$				Group III Small dose every other day $\times 5$	
	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding	3rd bleeding	4th bleeding	Pre-vacc.	2nd bleeding
Under 4 yrs.	<32 (18)*	256 (18)	1024 (18)	1024 (18)	<32 (17)	92 (17)	409 (17)	333 (14)	<32 (18)	512 (18)
4 years or older	32 (12)	1792 (12)	1536 (12)	1024 (12)	38 (11)	128 (11)	409 (10)	435 (11)	38 (11)	592 (11)

* The numbers in parentheses represent number of individuals from which the means were calculated.

high titers fell somewhat, and the lower titers were elevated. The grouping tendency noted above has been reported before (2). However, the fact that repeated injections of vaccine most frequently affected titers of low value was not pointed out. The most pronounced demonstration of this convergence effect can be seen in the Group I sera in Figure 1.

The titers reached against the heterologous type A strains largely followed the same pattern of development as observed with the homologous strain. Moreover, the antibody response appeared to diminish as the antigenic relationship of the heterologous strains to the PR8 strain receded. The titers obtained against swine virus were not impressive and measurements of the sera against swine strains are not included in the selected group listed in Table V. The quantitative aspects

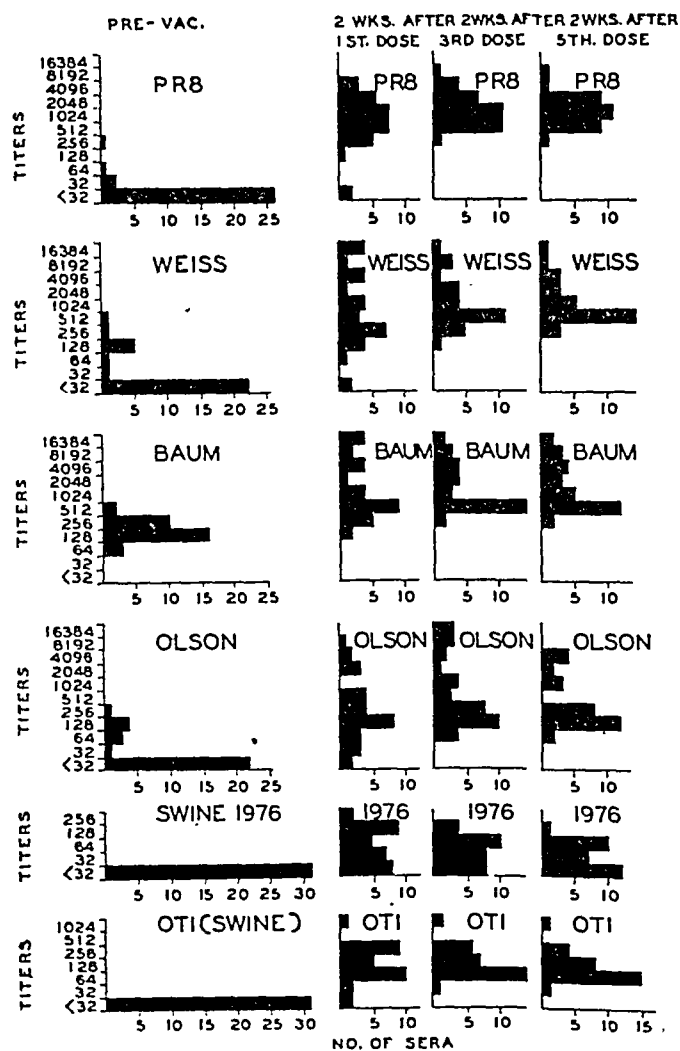


FIG. 1. DISTRIBUTION OF SERUM TITRATIONS IN GROUP I AGAINST TEST STRAINS

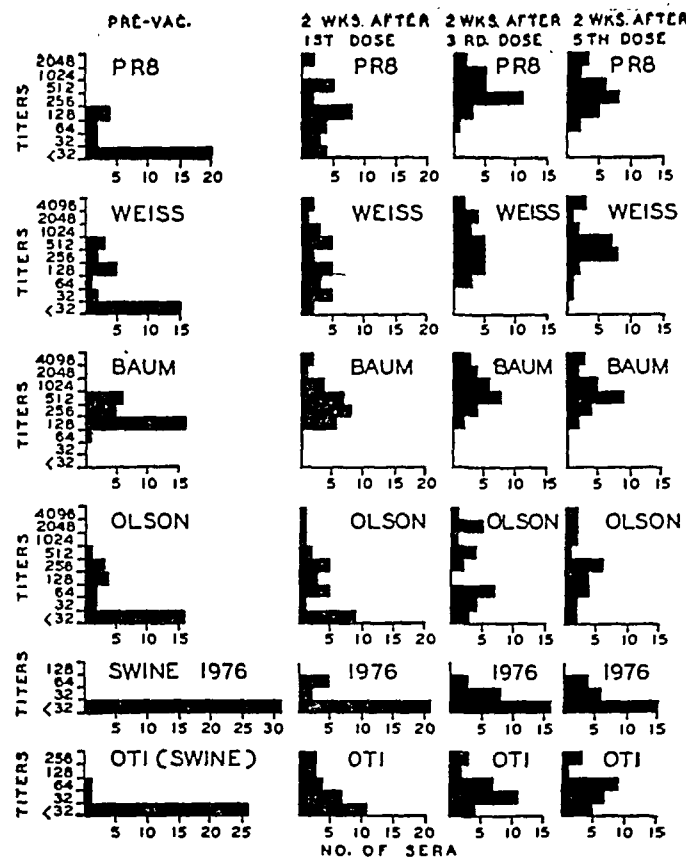


FIG. 2. DISTRIBUTION OF SERUM TITRATIONS IN GROUP II AGAINST TEST STRAINS

of the antibody responses to the heterologous as well as to the homologous strain are related to dosage (Table IV).

In Group III small doses given every other day for five days resulted in higher titers than in Group II where the same dose was given at two-week intervals. The mean titers for the former are quite similar to those in Group I after the first dose. The amounts of inactivated virus given in the first dose to Group I and in the five doses to Group III are roughly 5:3. So the use of $\frac{3}{5}$ as much vaccine given in repeated small doses resulted in as good a response as the use of a single large injection, and with the latter reactions were more frequent. It appears that maximal response with minimum reaction lies somewhere between the programs of Groups I and III.

DISCUSSION

The present data obtained in groups of infants and young children show that: (a) with a single large dose of influenza virus vaccine the mean antibody titer to the homologous strain was significantly increased; (b) two further injections of

vaccine resulted in cumulative increases in mean antibody titer; (c) this finding was most evident in children between the ages of one and three and a half years; and (d) where the effect was measurable the use of more than three doses contributed no significant advantages to antibody production.

The above results for the homologous strain were paralleled by the other type A strains tested. The efficiency of multiple injections of the PR8 strain in inducing antibody response to heterologous strains was fairly good with other type A strains and the degree of response appeared to vary with the test strain. Antibody response against two swine strains was meager. However, higher mean levels of antibody to all the strains studied were associated with multiple injections of the PR8 strain except in the children of four years or more who received the large doses; in these instances, the complete effect was obtained with one injection.

Other reports of the antibody response to influenza virus vaccine (3, 16 to 21) have indicated that maximum titers occur with a single injection of vaccine and that further injections did not increase the height of the titer. However, most of these studies were conducted in older subjects.

It should be pointed out that the interval between the second and third bleedings in Group I was 27 days and that the third bleeding was six weeks after the first dose of vaccine. It is possible that the results obtained in the third bleeding represent continued rise associated with stimulating effect caused by the initial injection. In some individuals, however, no increase in titer was observed two weeks after the first dose suggesting that the subsequent rise in their titers was related to the additional inoculations. This effect of delayed rising of titers was noted more strikingly in Group II. Nevertheless, the data here do not permit a precise conclusion. Further investigation of the time relation of antibody formation in infants and children must be done to clarify this point. The rises in titer against the heterologous type A strains can be postulated as being due to common antigenic characteristics.

The close agreement in mean response of all groups when tested against the PR8 and Weiss strains points to the use of only one of these strains in a vaccine. Recent experience with an outbreak of type A influenza virus infection in the late winter and spring of 1947 (22 to 24) indicates that the PR8 and Weiss strains did not have antigenic characteristics sufficiently broad to

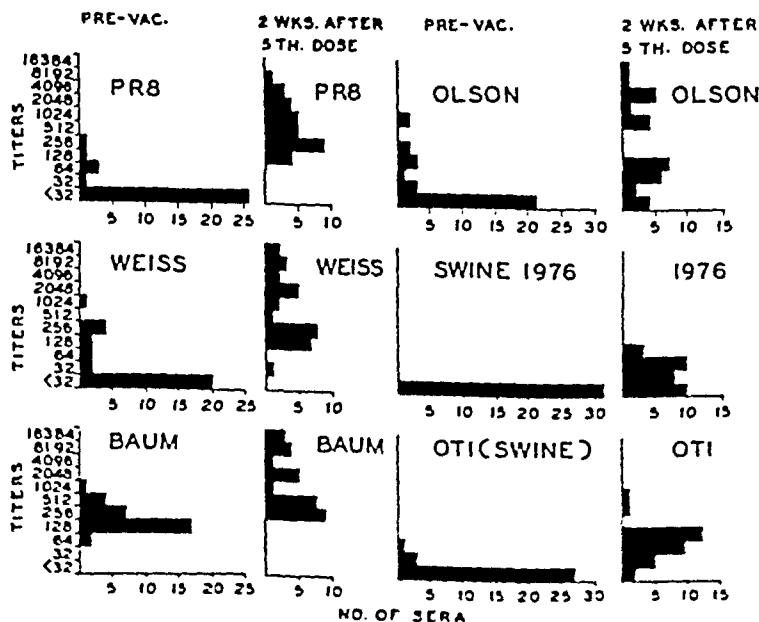


FIG. 3. DISTRIBUTION OF SERUM TITRATIONS IN GROUP III AGAINST TEST STRAINS

stimulate the formation of antibodies against the new strains when a single subcutaneous injection of vaccine was given. Accordingly, the sera of the selected members from Group I were tested against a strain of type A virus isolated in the early 1947 epidemic (Rhodes). Sera from five of the 19 individuals tested had no rise in antibodies between the prevaccination bleeding and the bleeding taken two weeks after the last dose; four had two-fold rises, and 10 had four-fold or greater rises in titer. However, the highest titers reached were only 512 in four instances. The remainder were 256 or less. Thus repeated vaccinations with the PR8 strain did result in the development of antibodies to the 1947 strain in some of the children.

Other than antigenic specificity, the relation of dose, number of injections, and interval between injections requires some further consideration. It is apparent, from the presented data, that maximal antibody response in children does not always occur with a single injection of vaccine. Despite the fact that a maximal dose, in terms of reactions to the vaccine, was given to the children in Group I, subsequent injections caused a further elevation in titer. This finding was most pronounced in the children under four years of age (Table VI). The amount of vaccine in the first dose was roughly equivalent to 1 ml. of infected allantoic fluid. The later injections approximated 0.65 ml. of allantoic fluid. With two more injections of the latter amount significant rises occurred in the mean titers against all type A strains tested. Decreasing the dosage to a concentration equivalent to 0.15 ml. of infected allantoic fluid, as in Groups II and III, gave lower mean antibody titers. However, using such a small dose the antibody response was greater than anticipated. The results of the Group III study, where the small amount of vaccine was given every other day for five days, showed a better mean titer than when given over the longer period of time as in Group II. As noted earlier the probable optimal dose and interval between doses lies somewhere between the effects noted in Groups I and III, also the use of more than three injections of vaccine did not contribute any significant changes. A reasonable schedule would seem to be the use of three injections of the equivalent of 0.3 ml. of infected unconcentrated allantoic fluid spaced at

weekly intervals. The use of concentrated material in infants and young children is not advisable unless the vaccine preparation is diluted prior to use.

SUMMARY

Multiple subcutaneous injections in children, whose average age was 3.2 years, of an influenza virus vaccine containing the type A PR8 strain, gave greater rises in mean titer of erythrocyte agglutination-inhibiting antibody against the homologous strain than did a single injection of the same virus preparation. In children of four years of age or older, who were living at the time of the last previous epidemic of influenza A in the area, the maximal response was obtained after a single large dose. Rises in titer to three other type A strains tested also occurred. Rises in titer to two strains of swine influenza virus were less pronounced. The height of antibody response to the heterologous strains was apparently influenced by the intimacy of their antigenic relationship to the PR8 strain and was not progressively enhanced as the administration of vaccine was continued beyond three doses.

The significance of the antigenic variation of strains, dosage, and schedule of injections of influenza virus vaccine in children is discussed.

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THE EFFECT OF SPONTANEOUS AND ARTIFICIALLY INDUCED FEVER ON LIVER FUNCTION

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Abnormal liver function has been reported in malaria (1-11), and in infectious mononucleosis (12), and its occurrence has been mentioned in pneumonia (13). Wade and Richmann (14) considered the possible influence of non-specific fever on the cephalin-cholesterol flocculation test, and Machella (15) has reported bromsulfalein retention during fever following foreign protein injection. Bragdon (16) recently reported a fatal case of post hypertherm hepatitis and others (17-25) have reported various degrees of liver damage associated with hyperpyrexia.

While on duty with the Eighth Evacuation Hospital of the Fifth Army in Italy during the winter of 1944-45 we had the opportunity to observe the behavior of several liver function tests in various disorders. Those observations indicated that abnormal results occurred frequently in conditions other than infectious hepatitis and led us to suspect that fever exerted an important influence on the rate of bromsulfalein clearance. This suggested the importance of studying the effect of artificially induced fever on liver function. Such a study has been completed on patients on the wards of the University of Virginia Hospital. The observations made on Army personnel with various diseases and those made on patients with induced fever are reported in this communication.

METHODS

In the subjects with various diseases the choice of tests was limited by the circumstances under which the studies were performed. The following procedures were employed:

1. The bromsulfalein liver function test as modified by Mateer *et al.* (26) in which 5 mgm. of dye per kilogram of body weight are given intravenously and the percentage retention determined in a single blood sample after 45 minutes.

2. Cephalin-cholesterol flocculation test: method of Hanger (27). Normal serum was always used as a control.

In the individuals in whom fever was induced by artificial means the following observations were made in each subject:

1. The bromsulfalein clearance (26);
2. Cephalin-cholesterol flocculation of Hanger (27);
3. Prothrombin time; Quick (28);
4. Total plasma protein by the copper sulfate method (29);
5. Icterus index using potassium dichromate standards;
6. Plasma bilirubin level (30);
7. Hematocrit, erythrocyte count, and hemoglobin determination (31);
8. Repeated measurements of the pulse, blood pressure, skin temperature and rectal temperature.

MATERIAL

The patients with clinical diseases were divided into four groups:

Group I: Acute infectious hepatitis with jaundice. One hundred and twenty patients in this group had overt jaundice at the time of admission to the hospital. The average duration of symptoms before admission was 8.0 days.

Group II: Tertian malaria. This group was composed of 100 patients treated in the hospital for malaria. In each instance the diagnosis was established by the finding of *plasmodium vivax* in the peripheral blood.

Group III: Primary atypical pneumonia. There were 100 patients in this group. In each instance the history, clinical course, leukocyte count, and chest roentgenogram were consistent with the diagnosis. The average duration of fever was 9.3 days and the average period of hospitalization of 50 unselected cases was 23.0 days. (It has been shown that "Q" fever was prevalent in the region at the time this study was carried out, and it is possible that some cases of this disease were included in Group III.)

Group IV: Miscellaneous conditions. This group consisted of 51 patients who gave a negative history of jaundice, malaria, infectious mononucleosis, and pneumonia, and had no evidence of these diseases during the period of observation. Of these, 37 were patients without fever who were diagnosed anxiety state, stabies, sprains (back, knee, ankle), acute gonorrheal urethritis, and epididymitis, acute and recurrent. The remaining 14 patients had fever due to acute tonsillitis, lobar pneumonia, acute epididymitis, acute appendicitis, cellulitis of jaw, wounds of the extremities, acute gastroenteritis and

bacillary dysentery. Fevers of unknown origin were not included.

In the study of the effect of artificially induced fever, 12 white subjects of both sexes, ranging in age from 16 to 50, were used. These patients with syphilis, arthritis, chorea, or psychoneurosis were subjected to 26 bouts of fever induced by the intravenous injection of killed typhoid organisms. These patients were not denied water and were given salt in the form of tablets at regular intervals during the febrile period. Liver function tests were performed before injection of the foreign protein and were repeated three to four hours later when fever had been present for about two hours. In nine individuals liver function tests were repeated within 24 hours after fever had subsided. Sample observations are shown in Figure 1. The control studies were performed on white and colored subjects of both sexes, ranging in age from 16 to 56.

RESULTS

Cephalin-cholesterol flocculation test

The percentage of patients in Groups II, III and IV showing positive flocculation tests is

TABLE I
Results of cephalin-cholesterol flocculation tests in patients with illnesses other than infectious hepatitis

Groups	Max. oral temperature day of test	Number of tests	Flocculation reading at 24 hours		
			++++ +++	++	+ 0
II	° F.		per cent	per cent	per cent
	103 or more	10	80.0	10.0	10.0
	99 ² -102 ⁸	8	100.0	0.0	0.0
III	103 or more	44	27.0	11.0	62.0
	99 ² -102 ⁸	39	20.0	18.0	62.0
	99 or less	71	52.0	11.0	37.0
IV	99 ² or more	14	28.5	28.5	43.0
	99 or less	37	5.9	8.1	86.0

shown in Table I. No correlation with the amount of fever is apparent. An unexplained but interesting observation is the higher incidence of abnormal cephalin-cholesterol flocculation in the

SUBJECT 3

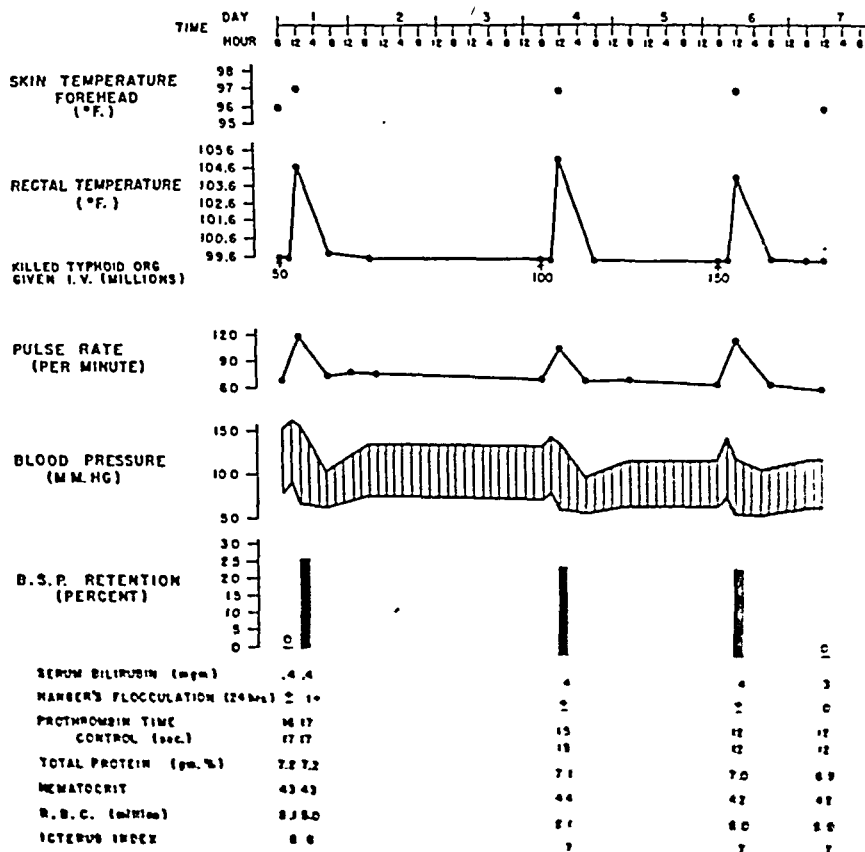


FIG. 1. SAMPLE CHART ILLUSTRATING INVESTIGATIONS ON EACH SUBJECT

TABLE II

Results of cephalin-cholesterol flocculation test in atypical pneumonia and infectious hepatitis

Week of disease	Atypical pneumonia				Infectious hepatitis			
	Number of tests	Flocculation reading 24 hours			Number of tests	Flocculation reading 24 hours		
		++++ ++++	++	+ 0		++++ ++++	++	+ -
1	69	per cent 24.4	per cent 10.0	per cent 65.6	53	per cent 96.2	per cent 3.8	per cent 0.0
2	77	27.3	18.1	54.6	71	91.0	7.5	1.5
3	58	58.0	12.0	30.0	12	75.0	25.0	0.0
4	30	76.0	7.0	17.0				

post-febrile period in the atypical pneumonia group. Table II compares the percentage of positive tests in atypical pneumonia (Group III) and acute hepatitis (Group I) with reference to the weeks after onset of the disease. It is apparent that the incidence of positive tests increases in the pneumonia group with the lapse of time while the hepatitis group shows no such change. In Figure 2 are shown the results of the cephalin-cholesterol flocculation tests with reference to day of disease in three patients with atypical pneumonia. In a majority of these patients, we were unable to determine the length of time that the flocculation reaction persisted. In 19 patients the test was performed between 30 and 50 days after onset and a 3 plus reaction at 24 hours occurred in 14 patients, a 2 plus reaction in one.

Bromsulfalein test

Bromsulfalein tests were not performed in patients with jaundice (Group I). The relationship between the amount of fever in the patients in Groups II, III and IV and the percentage of these patients showing an abnormal bromsulfalein retention is presented in Table III. Abnormal dye retention occurred much oftener in each group when fever was present. No patients with malaria showed any dye retention when the test was performed two days or more after the termination of fever. Figure 3 illustrates the relationship of fever and bromsulfalein retention in an individual with atypical pneumonia.

Of the tests related to liver function, only the bromsulfalein test showed significant variation in subjects with fever induced by artificial means.

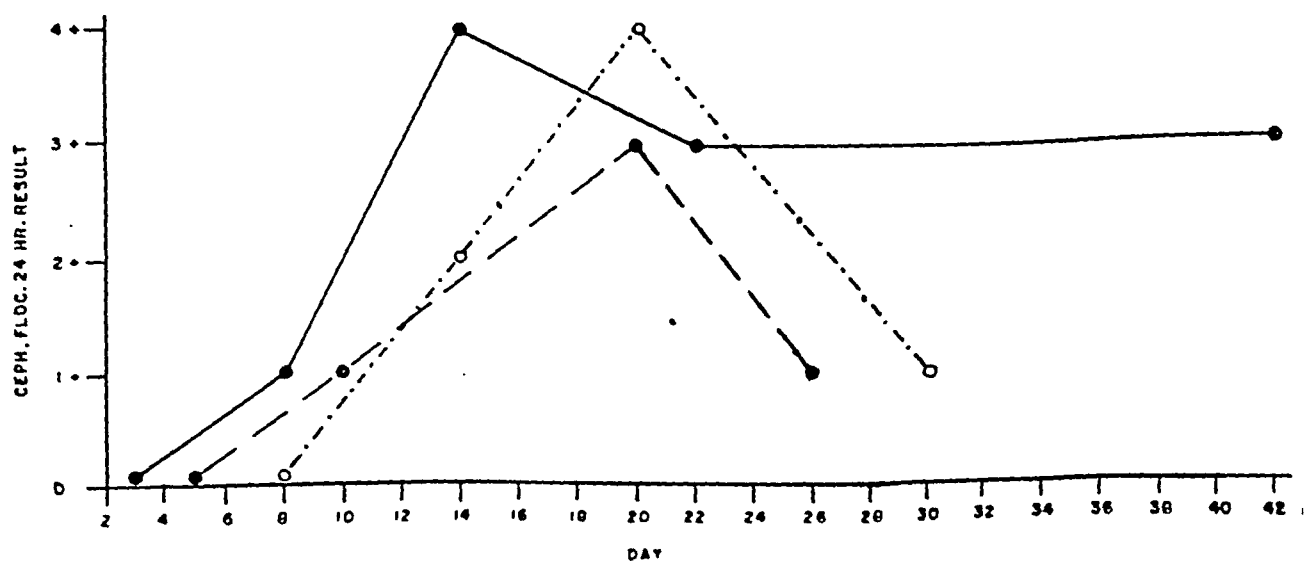


FIG. 2. CEPHALIN-CHOLESTEROL FLOCCULATION TESTS IN THREE PATIENTS AT DIFFERENT TIMES AFTER ONSET OF ATYPICAL PNEUMONIA

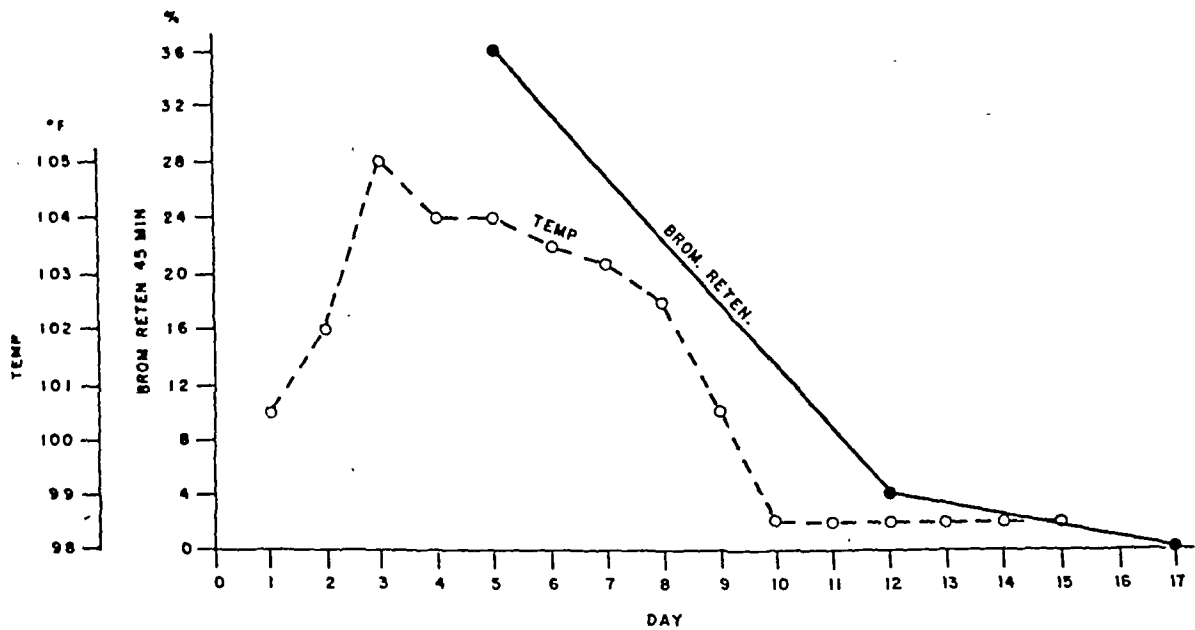


FIG. 3. BROMSULFALEIN TESTS AND TEMPERATURE CURVE IN A PATIENT WITH ATYPICAL PNEUMONIA

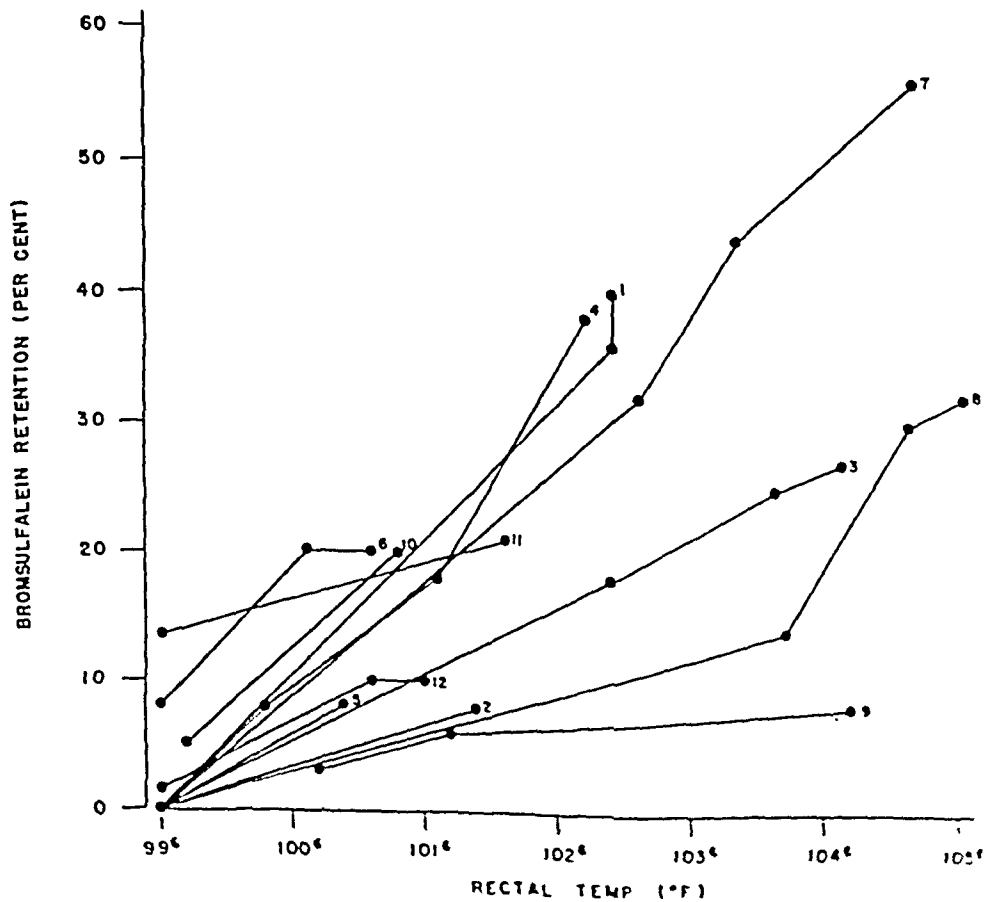


FIG. 4. BROMSULFALEIN TESTS IN 12 PATIENTS WITH FEVER INDUCED BY INJECTION OF FOREIGN PROTEIN

decrease in the flocculation-inhibiting property of the albumin. Dole and Emerson (35) found that the gamma globulin was increased and the albumin was decreased in the electrophoretic pattern of patients with malaria. No electrophoretic studies of serum of patients convalescing from atypical pneumonia have been found, but it seems likely that an increase in gamma globulin occurs if immune bodies are formed. An increase in gamma globulin might explain the increasing percentage of positive flocculation tests observed during the convalescent phase.

Although the patients with various diseases showed abnormal BSP retention much more often on days when fever was present, the time that the test was performed did not necessarily coincide with the peak of temperature elevation. The results obtained with induced fever suggest that more detailed observation may have shown even better correlation between the temperature and dye retention.

The subjects who developed fever following foreign protein injection showed impaired ability of the liver to clear bromsulfalein. In patients with fever induced by the application of external heat, similar changes in clearance of the dye occurred. The administration of foreign protein which was not followed by temperature elevation, was not associated with impaired dye clearance. These results indicate that the fever rather than the foreign protein was the factor responsible for the dye retention. Since aminopyrine prevents the febrile response to foreign protein injection but does not prevent an increase in the blood flow to the kidney and liver, it appears probable that the diminished clearance of the dye observed in this study is due to impaired function of the cells in the liver rather than to changes in the hepatic circulation. The fact that in about one-half of the subjects with induced fever bromsulfalein clearance was impaired for 24 hours after fever subsided, favors this interpretation. In view of the jaundice and marked histologic change in the liver that has been reported after extreme hyperpyrexia (16-25) it appears probable that lower degrees of fever, such as encountered in this study, are associated with similar but less marked changes in the liver.

CONCLUSIONS

1. Abnormal bromsulfalein retention and abnormal cephalin-cholesterol flocculation tests occur frequently in disorders other than infectious hepatitis.
2. In a group of patients with atypical pneumonia, the highest incidence of abnormal cephalin-cholesterol flocculation reactions occurred during convalescence.
3. In all groups that were studied the incidence of abnormal bromsulfalein retention was much higher on days when fever was present.
4. Fever induced by artificial means was associated with diminished bromsulfalein clearance. For reasons presented in the discussion, it appears probable that diminished bromsulfalein clearance is due to impaired function of the cells in the liver.
5. The temperature of the patient at the time the test is performed must be considered in the interpretation of the bromsulfalein test.

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THE EFFECTS OF THE CARDIAC GLYCOSIDES UPON THE DYNAMICS OF THE CIRCULATION IN CONGESTIVE HEART FAILURE. I. OUABAIN^{1, 2}

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INTRODUCTION

While the clinical recognition, and even quantitative appraisal of congestive heart failure is a matter of routine in the practice of medicine, no precise definition of this state has been formulated which is equally satisfactory to clinicians, physiologists and pathologists. There seems little doubt that among the many aspects of the circulation which may be affected when cardiac function is abnormal, no single abnormality is always the major or primary factor, nor need any single one invariably be present. This is true for changes in cardiac output, blood volume, sodium retention, diastolic heart volume, mechanical efficiency of the heart, peripheral venous pressure, intracardiac pressures, and peripheral vasomotor reactions. It is nevertheless true that clinically the diagnosis of heart failure is made by the detection of signs and symptoms referable to abnormal increases in the volume of blood in various parts of the circulation—and the assumption is made that these increases are the result of aberration in cardiac function.

Equally a matter of routine in clinical medicine is the administration of a cardiac glycoside, either in a relatively pure state or more often as digitalis leaf, in the justified expectancy that it will serve to help dispel and prevent recurrence of congestive heart failure. Yet, in spite of a voluminous literature upon the subject, there is still great diversity of opinion as to the site and mode of action of these drugs upon the circulation, especially in human beings, and as to differences among the individual drugs in these actions.

The object of the present study is twofold: first, by the application of certain newer techniques, to gain further insight into the action of various cardiac glycosides in patients with congestive heart failure; second, to gain information as to causal relationships between the various circulatory abnormalities, from a study of the emergence of patients from congestive failure.

Thirteen patients with heart disease, with and without clinical evidence of congestive failure, were chosen who had not received any cardiac glycoside within three weeks. The one exception to this was Patient No. 12, who was given 0.6 mg. of ouabain 12 hours before the control observations reported. Most of them were studied within 12 to 24 hours after admission to the hospital. Therapy had consisted of rest, sedation and occasionally oxygen. Patients were studied fasting, and wherever compatible with their comfort, in the recumbent position. While circumstances were often not conducive to a truly basal state, a preliminary oxygen consumption was done under conditions of quiet, for purposes of comparison with later determinations. Catheterization of the right heart was performed with a single-lumen catheter, using the method of Cournand and Ranges (1-3), and the catheter was guided into the right ventricle or pulmonary artery under fluoroscopic visualization.⁴ In one case, auricular catheterization only was possible. An inlying needle was placed in one femoral artery, and in nine patients in a peripheral arm vein. Electrocardiographic leads were attached in all cases. The catheter was kept patent by a slow, continuous drip of a 5 per cent solution of glucose in distilled water, to 1500 cc. of which 5 mg. of heparin was added. Cardiac outputs were done by the direct Fick method. Mixed venous blood samples were taken from the right ventricle except in Patient No. 7 where an auricular sample was used; blood oxygen contents were measured by the Van Slyke technique. Oxygen consumption during the blood sampling was determined graphically on a metabolism machine, using two- to four-minute breathing periods as the basis for computing the minute uptake. Intracardiac, femoral arterial and peripheral venous pressures were optically recorded through

¹ The expenses of this investigation were supported by a grant from the Life Insurance Medical Research Fund.

² Read at the Meeting of the American Society for Clinical Investigation, Atlantic City, May 5, 1947.

³ Post-graduate Research Fellow, Life Insurance Medical Research Fund.

⁴ Assistance in fluoroscopic visualization was given by the X-ray Department (Dr. Max Ritvo, Director).

Hamilton manometers. The zero level for pressures in the peripheral vein and lesser circuit was taken as 5 cm. below the angle of Louis. Femoral systolic and diastolic pressures were obtained by averaging the measured values of a representative sequence of beats in one or more respiratory cycles. Right ventricular systolic and pulmonary arterial systolic and diastolic pressures were similarly averaged. Femoral mean, pulmonary mean, right auricular and peripheral venous pressures were measured by planimetric integration. The diastolic pressure in the right ventricle was measured at the moment of onset of the systolic rise, since this figure was held to be most representative of the filling pressure at which systole began (4), and hence of the tension within the ventricular myocardium at its beginning of contraction. Pulse pressures in the femoral and pulmonary arteries were measured as the difference between the systolic and diastolic values. The right ventricular pulse pressure was measured as the difference between the systolic and the end-diastolic pressures. The peripheral arterial resistance was computed in accordance with the formula:

$$R = \frac{Pm \text{ (mean pressure in mm. Hg)} \times 1332}{C.O. \text{ (cardiac output in cc./sec.)}} \quad (5)$$

Sterile solutions of ouabain were injected through the catheter over a period of one to two minutes in doses of 0.25 to 0.75 mg.

In nine patients, a cardiac output was done before giving ouabain, and at the end of the observation period, which varied from about 40 to about 160 minutes. In one patient studied early in the series, before it was realized how quickly significant data could be obtained, both outputs were done after the administration of ouabain. In three patients (Nos. 11, 12, 13), in addition to a control, serial cardiac outputs were done after they had received the drug, at the times noted on the charts. In each instance pressures were recorded as close to the time of the outputs as possible and at varying intervals between.

CASE MATERIAL

Thirteen patients were studied. The diagnoses and clinical state of compensation are listed below.

(a) In clinical congestive failure—10

Arteriosclerotic heart disease	4
Hypertensive heart disease	3
Hypertensive and arteriosclerotic heart disease	2
Cor pulmonale	1

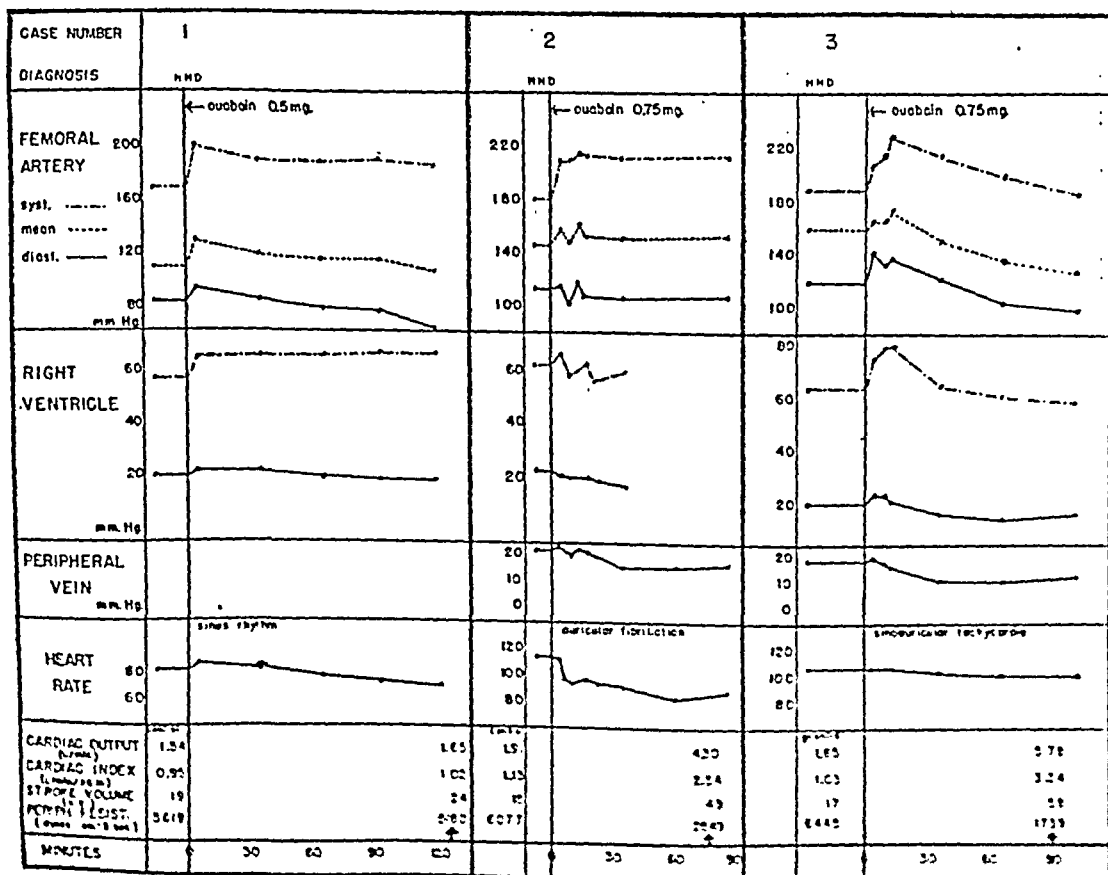


FIG. 1. THREE PATIENTS WITH HYPERTENSIVE HEART DISEASE IN CLINICAL HEART FAILURE, WITH INCREASE IN CARDIAC OUTPUTS AFTER OUABAIN

(b) *Not clearly in clinical congestive failure—3*

Arteriosclerotic heart disease	1
Rheumatic heart disease with mitral stenosis	1
Heart disease of unknown etiology, ? beriberi	1

Four of the patients in Group (a) and two of those in Group (b) had auricular fibrillation.

RESULTS

Cardiac output. An increase in cardiac output was noted in ten patients, including nine of those in obvious congestive failure. A decrease in output was observed in two patients, neither of whom was in clinical failure. No significant change took place in one patient who was in heart failure.

Pressure changes. Significant pressure changes

following ouabain were noted within one to eight minutes in ten of the 12 studies in which control data were obtained prior to the drug's administration. One exception was Patient No. 7 who received the small dose of 0.25 mg. At five minutes his pressures were unchanged; the next observations were made at 35 minutes. The other exception was Patient No. 10 whose first post-ouabain measurements were not made until 27 minutes, at which time changes were present. In Patient No. 8 the first measurements were made 46 minutes after ouabain was given; at the time of the next observation—27 minutes later—the changes shown on his chart were found.

The multiplicity of changes which had occurred by the end of the experimental period made it ap-

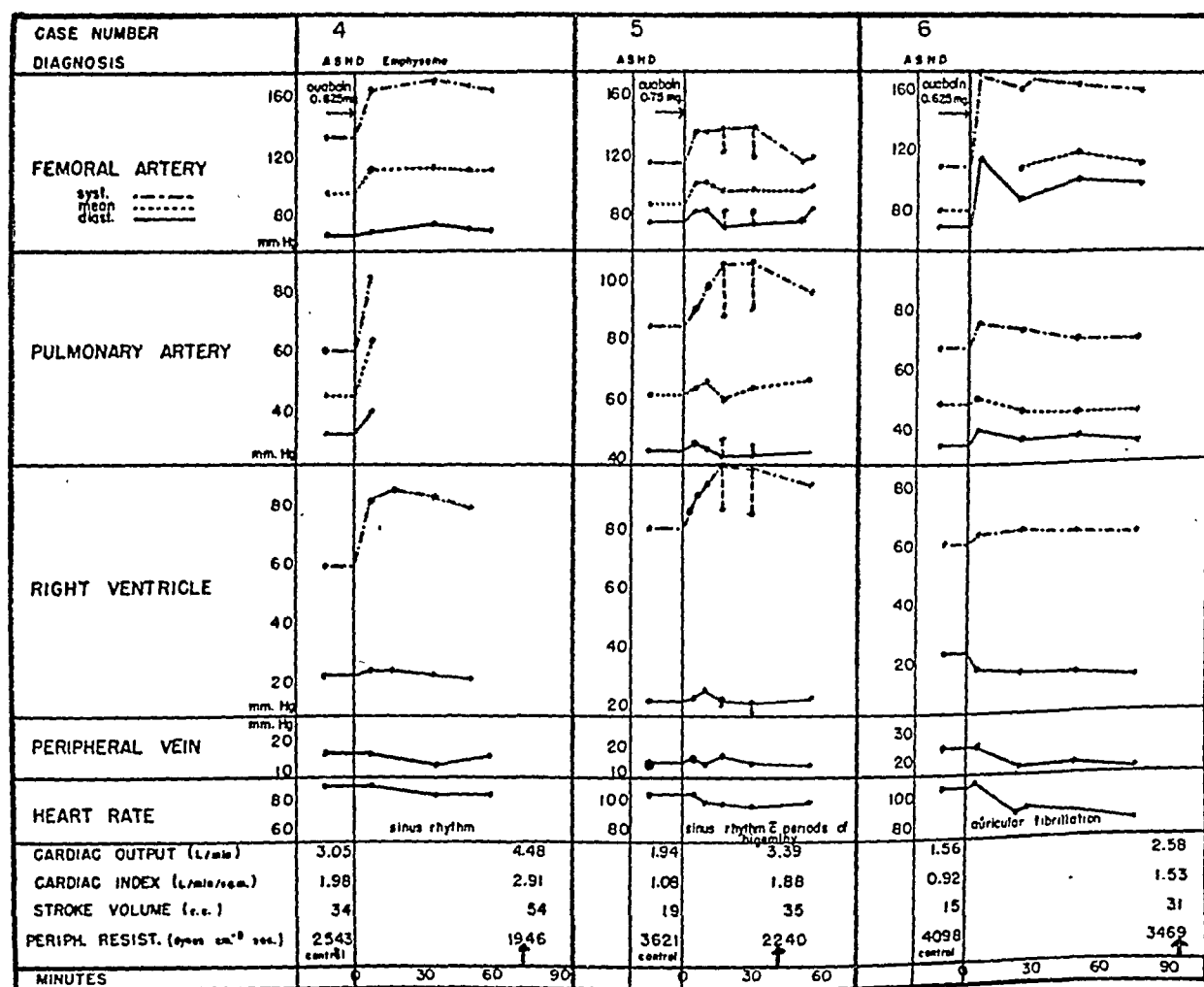


FIG. 2. THREE PATIENTS WITH ARTERIOSCLEROTIC HEART DISEASE IN CLINICAL HEART FAILURE, WITH INCREASE IN CARDIAC OUTPUTS AFTER OUABAIN

In Case No. 5 during periods of bigeminy the pressures of ectopic beats are charted separately and connected to the pressures of normal beats by vertical broken lines. In Case No. 6 the discrepancy between pulmonary arterial and right ventricular systolic pressures was due to oscillatory artefacts which rendered inexact the measurement of the point of maximum pressure.

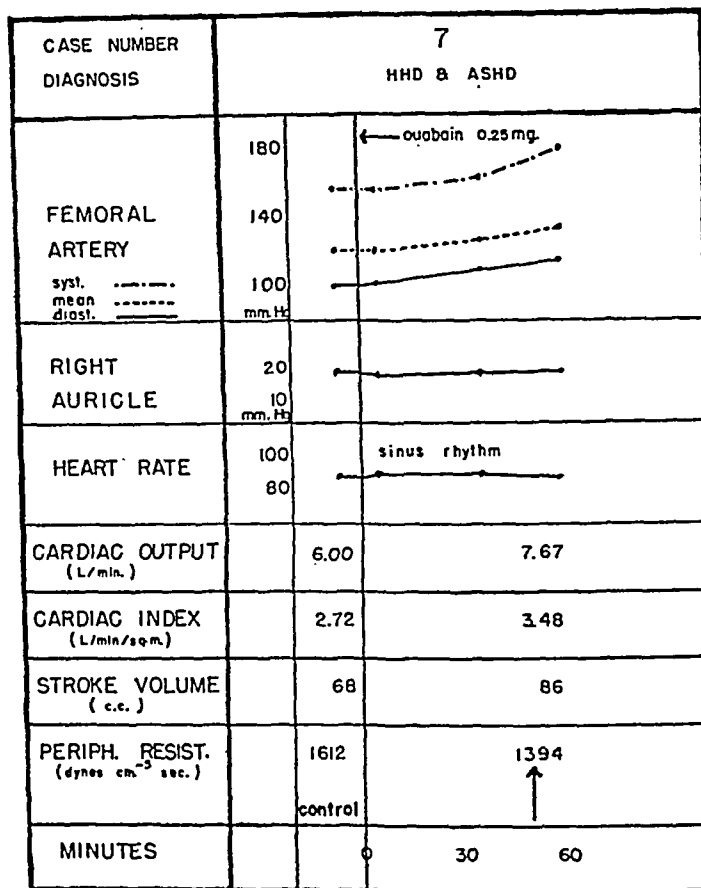


FIG. 3. PATIENT WITH HYPERTENSIVE AND ARTERIOSCLEROTIC HEART DISEASE IN CLINICAL HEART FAILURE, WITH INCREASE IN CARDIAC OUTPUT AFTER OUABAIN

parent that the site and mode of action of ouabain could only be seen from the actual sequence in which such changes developed. For purposes of clarity the complete data from each case are shown graphically.

A. Early changes.

1. In the course of a rising cardiac output.

(a) Patients in clinical congestive failure: A striking uniformity of response can be seen in the nine patients who were in failure (Nos. 1-7; 11, 12). All initially showed elevations of pressure in the right heart and pressure pulse contours of a type previously reported as found in right heart failure (4). In every instance following ouabain, femoral arterial systolic pressures promptly rose as did the mean and pulse pressures; this occurred, however, considerably later

in Patient No. 7 who received the smallest dose. In the eight on whom records were taken, right ventricular systolic and pulse pressures also increased (Nos. 1-6; 11, 12). In Patient No. 6 technical difficulty made impossible the measurement of femoral mean pressure at the time the other earliest changes were seen; at the time of the next observations, the femoral mean pressure was found to have risen markedly. In the three instances in which it was possible also to measure pulmonary arterial pressures both systolic and pulse pressures were similarly increased (Nos. 4-6; Figure 8). While ventricular diastolic pressures varied in degree of response, a significant drop was noted only in the ventricular diastolic pressure of Patient No. 6; here, too, however, the increased pulse pressure was accompanied by an increased systolic pressure. In the seven cases with recorded peripheral venous pressures no sig-

nificant change was seen up to the time at which these other phenomena had become apparent (Nos. 2-6; 11, 12). In the two patients lacking peripheral venous pressure data, the auricular pressure was unchanged in one (No. 7), while the ventricular diastolic pressure actually rose slightly in the other (No. 1). At the time that pressure changes already were manifest, the heart rate had not altered in five patients (Nos. 3-5; 7, 12); in two, the rate had risen by four beats per minute (Nos. 1, 6), and one of these had auricular fibrillation. The other two patients with auricular fibrillation exhibited a decline in ventricular rate from 114 to 97 in the first six minutes in Patient No. 2, and from 140 to 130 in three minutes in Patient No. 11.

(b) Patients not in clinical congestive failure: In the one patient not clearly in heart failure in whom the cardiac output rose (No. 8), the only significant change was a decline of 5 mm. Hg in the initially normal ventricular systolic pressure.

II. In the course of a falling cardiac output.

Patient No. 10 showed a small diminution in femoral systolic and diastolic pressures, with a slight decrease in the pulse pressure; the right ventricular pulse pressure widened by 4 mm., as a result of a slight rise in the systolic and a slight fall in the diastolic pressures. The fall in output was due almost wholly to a drop in heart rate, as

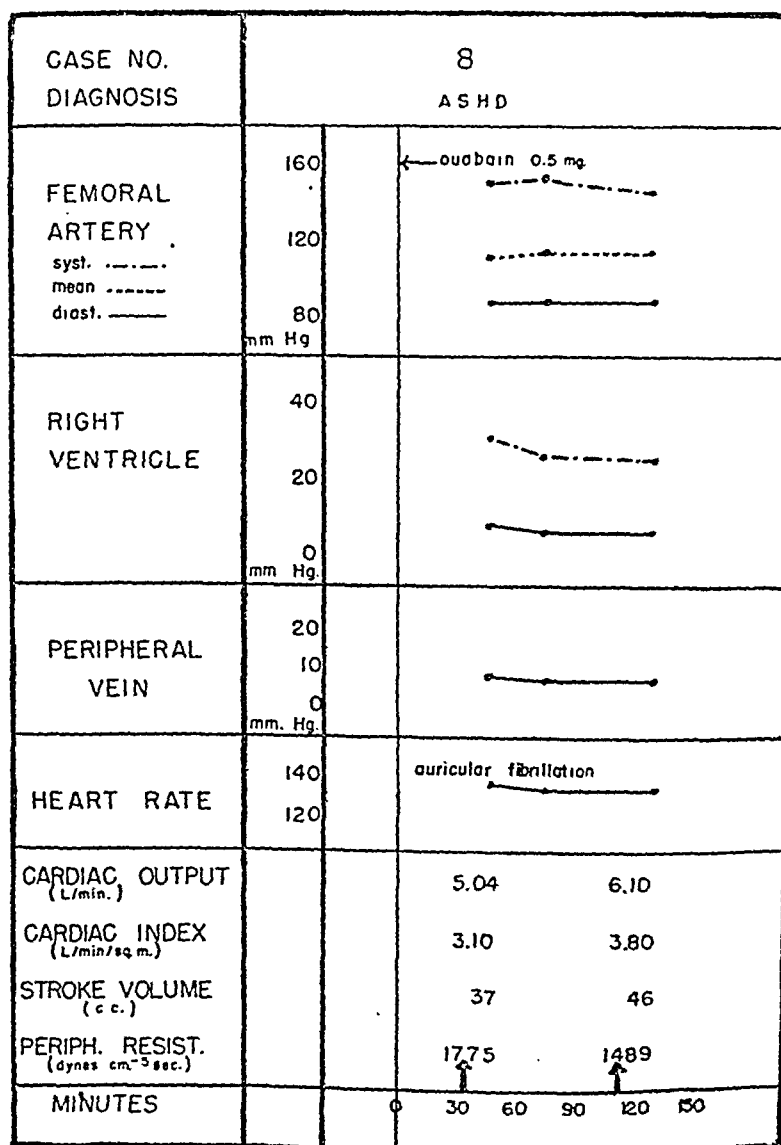


FIG. 4. PATIENT WITH ARTERIOSCLEROTIC HEART DISEASE NOT IN CLINICAL HEART FAILURE, WITH INCREASE IN CARDIAC OUTPUT AFTER OUABAIN

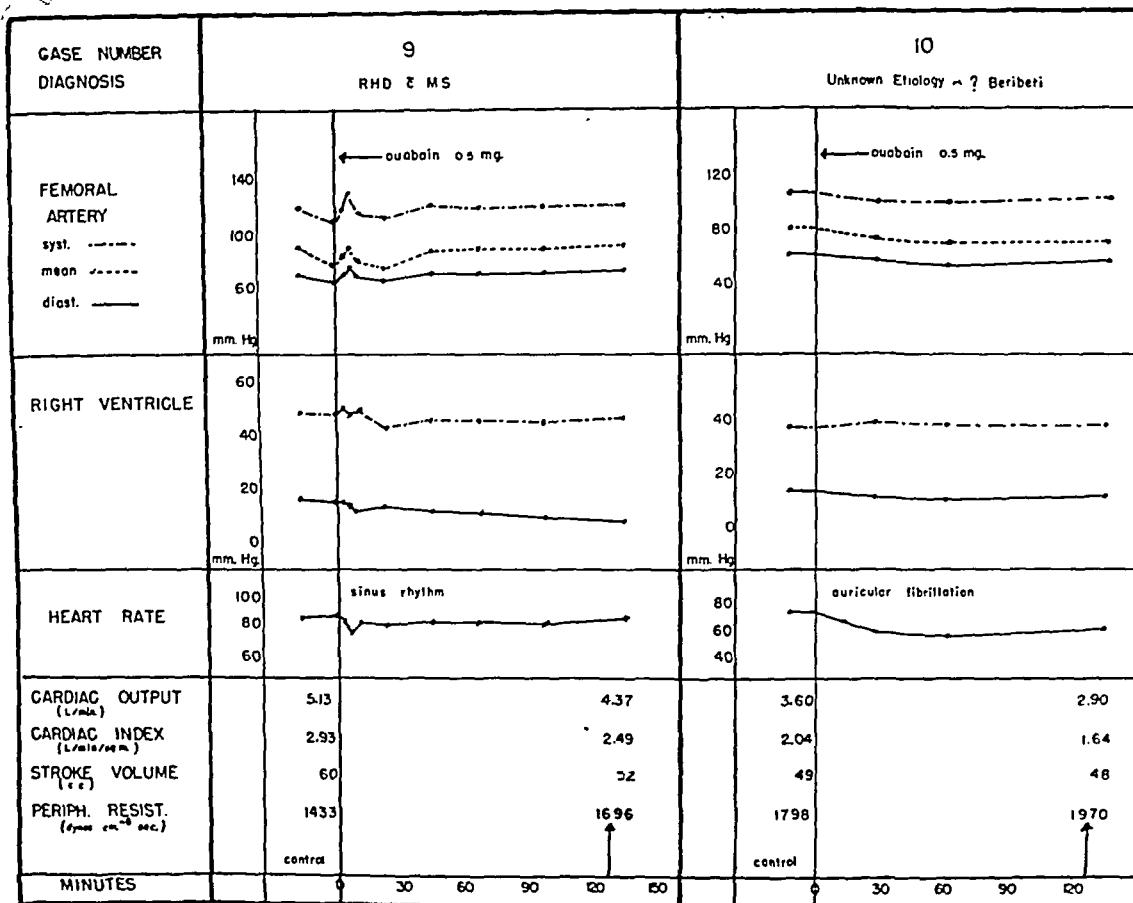


FIG. 5. TWO PATIENTS NOT IN CLINICAL HEART FAILURE, WITH DECREASE IN CARDIAC OUTPUTS AFTER OUABAIN

seen by the stroke volume which changed by only 1 cc., from 49 to 48.

The second patient whose output fell (No. 9) showed a definite early increase in femoral systolic and diastolic pressures, with a moderate increase in the pulse pressure; the right ventricular pulse pressure rose 4 mm., as a result chiefly of the fall in diastolic pressure. At this time his heart rate had decreased by 13 beats per minute.

In neither case was the peripheral venous pressure measured.

III. In the absence of a change in cardiac output.

In Patient No. 13 the changes in cardiac output were of an order so small that no action of ouabain can be held accountable for them. Five minutes after the drug was given the femoral systolic, diastolic and mean pressures had all risen to the

same degree, with no resulting alteration in pulse pressure. At ten minutes these values showed a decline toward control levels. During this interval the ventricular systolic and diastolic, and peripheral venous pressures, as well as the heart rate, were essentially unaltered.

B. Later changes.

As seen in the figures there was considerable variation in the degree to which the early changes described were maintained over the remainder of the experiments.

Of the nine patients in congestive failure showing rises in cardiac output, seven maintained a femoral systolic pressure increase (Nos. 1, 2, 4, 6, 7, 11, 12), while two returned to approximately control levels (Nos. 3, 5). Three maintained their early diastolic arterial pressure rise (Nos. 5-7). Only one, however, failed to keep an in-

creased arterial pulse pressure (No. 5). Five of the eight patients with ventricular pressure records maintained their early systolic increase (Nos. 1; 4-6; 12); only four showed a significant fall in diastolic pressure (Nos. 2, 3, 6, 11); all but two (Nos. 3, 11) maintained a widened ventricular pulse pressure. Only two of the pulmonary artery studies were followed throughout the experiment (Nos. 5, 6). In both the initial increase in pulse pressure was present to the end; in one the early slight diastolic pressure rise was followed by a fall to the control value. The peripheral venous pressure fell in five cases (Nos. 2, 3, 6, 11, 12). In four also, there was a decline in heart rate by the conclusion of the observations (Nos. 1, 2, 6, 11). No change occurred in the auricular pressure in Patient No. 7.

In the patient not clinically in heart failure (No. 8), but whose cardiac output rose, no

further important change was noted in any of the subsequent measurements.

The patient with mitral stenosis and a fall in cardiac output (No. 9) showed a return to the range of his control values except for a continued decline of ventricular diastolic pressure. The other patient whose cardiac output dropped (No. 10) showed no significant further changes after the early ones described, except a continuing moderate fall in heart rate. In the patient (No. 13) whose cardiac output showed little variation there was a gradual return of femoral pressures to their control level. The other data showed no appreciable variation throughout the experiment.

DISCUSSION

Numerous articles have been published on the physiological and pharmacological effects of ouabain (6-9) as well as of the other cardiac glyco-

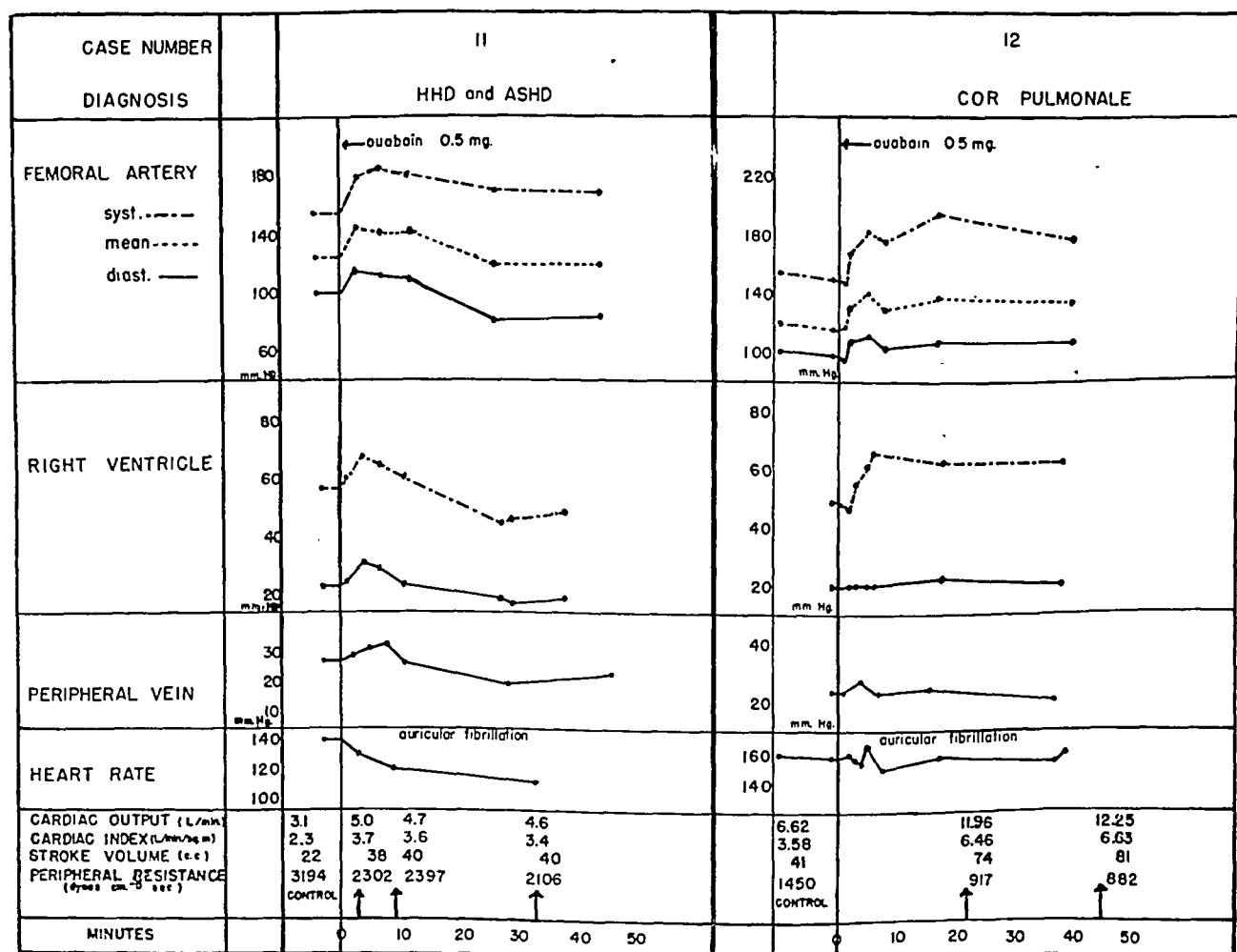


FIG. 6. TWO PATIENTS IN CLINICAL HEART FAILURE
Serial determinations show increases in cardiac outputs after ouabain.

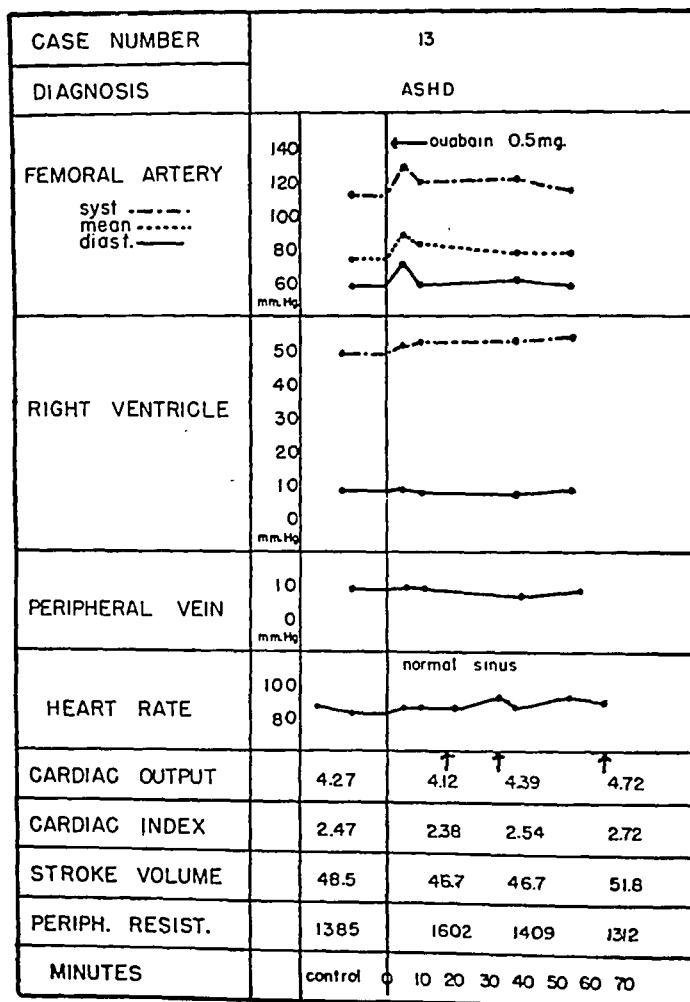


FIG. 7. PATIENT WITH ARTERIOSCLEROTIC HEART DISEASE IN CLINICAL HEART FAILURE

Serial determinations show no definite effect of ouabain on cardiac output.

sides. Summaries have been recently published by Freedberg and Zoll (10) as well as in the monograph by Movitt (11). In general it is believed that ouabain has an action on the circulation that is qualitatively similar to that of the digitalis glycosides and that the chief quantitative differences in its behavior are its rapidity of action, its rapid elimination, and its failure of absorption by the gastro-intestinal tract. It is the opinion (12) of some physiologists and clinicians, especially of the European and Latin American schools, that ouabain also differs from digitalis in having relatively less effect on the sinus node and junctional tissues but a more effective action on the cardiac musculature itself.

The pattern of early response in the nine patients in heart failure who exhibited a rise in cardiac output was so consistent that an identical effect of ouabain is suggested in them all. Their resting cardiac outputs were below normal, as shown by cardiac indices varying from 0.92 to 2.72 L. min. sq. m., with the exception of Patient No. 12 whose higher figure is consistent with his diagnosis of cor pulmonale (13, 14).² As seen from the data, the stroke volume rose in each instance under the influence of ouabain. That a decrease in filling, and hence a decrease in diastolic stretch "from behind," from the re-

² The average normal cardiac index is calculated to be $3.12 \pm .40$ L. min. sq. m. (3, 15).

ipheral venous pressure head, did not account for this stroke volume change seems established by the fact that the peripheral venous pressure had not decreased at the time of the first demonstrated increase in cardiac output in four of the seven subjects in which it was measured. In the two patients in whom this phenomenon was not studied, an equally valid index to ventricular filling was seen in the unchanged auricular pressure of Patient No. 7, and in the ventricular diastolic pressure of patient No. 1. In the three patients in whom the venous pressure had decreased at the time of the post-ouabain cardiac output determination, no change in this measurement had occurred earlier at a time when other marked circu-

latory effects were already evident in vascular pressure changes.

An effect on stroke volume and cardiac output through an action of ouabain on the ventricular rate seems poorly borne out by the lack of correlated change in seven instances; in Patient No. 11 where the rate had fallen at the time of the first measured rise in output it is noteworthy that the subsequent decline in rate was of the same order, but with a slight drop in output and no change in stroke volume. The only other mechanism, therefore, that can plausibly explain the increased stroke volume, is an increase in the vigor of ventricular systole as a result of the direct action of ouabain upon the contractility of the

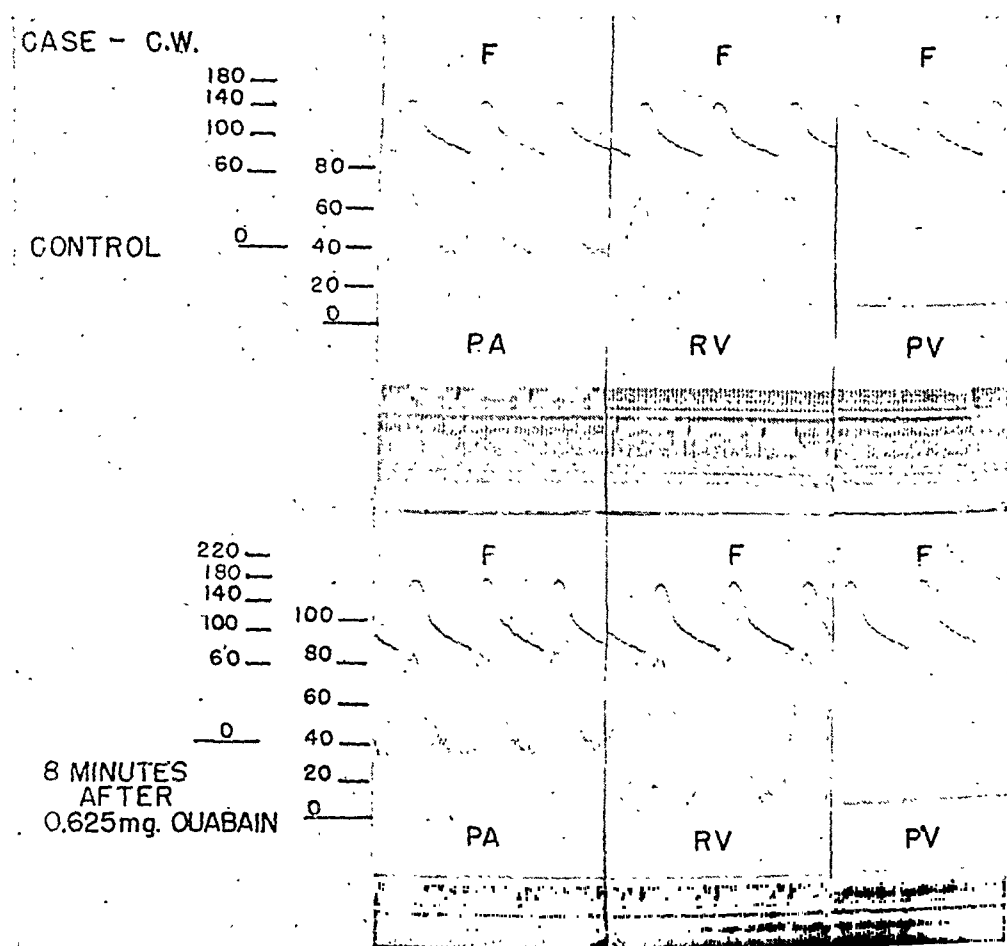


FIG. 8. TRACINGS OF CASE No. 4

The upper group shows control observations: simultaneous femoral arterial (F) and pulmonary arterial (PA) pressures; femoral arterial (F) and right ventricular (RV) pressures; and femoral arterial (F) and peripheral venous (PV) pressures. A simultaneous electrocardiogram is recorded in the lower part of each series. The pressure scales are in mm. of mercury, one for the femoral artery, one for the right heart and venous pressures. The two latter were done with the same manometer and from the same zero. The lower group shows corresponding tracings eight minutes after ouabain with the changes in pressures readily seen.

myocardium. The early pulse pressure changes are consistent with such a mechanism, and would indicate that it occurs very promptly after injection of ouabain. While changes in the peripheral resistance at this time, at both pulmonary and systemic arterioles, cannot be wholly excluded in those patients lacking early output studies after ouabain, the observed pressure changes are precisely what would be expected in the presence of a rapid and significant increase in cardiac output. In Patient No. 11 an increase in stroke volume and decrease in peripheral resistance were demonstrable within three minutes after the administration of ouabain at a time when the heart rate had fallen only from 140 to 130, and the increase in stroke volume was maintained at two further observations. In Patient No. 12, in whom the heart rate did not change, the increase in cardiac output and decrease in peripheral resistance observed at 19 minutes after giving ouabain were maintained at 45 minutes. The predominant role of the systolic component in the pulse pressure increase speaks, furthermore, chiefly for a stroke volume change, rather than for a change in peripheral resistance. Moreover, were the pressure rises due mainly to an increase in peripheral resistance, one would expect not only a more uniform finding of increased diastolic pressure, but also, in these already decompensated hearts, still further evidence of failure in the form of an increasing venous pressure and a decreasing cardiac output. The variability of the later findings, in which in some instances a return of pressures toward the control level occurred, does not argue against such an interpretation, in view of the likelihood that ultimately such other factors must come into play as changes in blood volume, heart rate, blood viscosity, etc. It is of interest, and indeed in keeping with the proposed explanation, that in every case in this group, regardless of the absolute values of the final measured pressures, the peripheral resistance was below its initial figure.

In Patient No. 8, who was not clearly in heart failure, the output increased between 32 and 110 minutes after ouabain administration. It is possible that earlier changes occurred, which took place before the observations were made. The patient was of particular interest for his unchanging ventricular rate of 133, in the presence of auricular fibrillation. While his initial right ven-

tricular systolic pressure was normal, the peripheral venous pressure was in the upper normal range, and the normal gradient between the venous and ventricular diastolic pressures did not exist—a finding which, as Richards *et al.* (16, 17) have pointed out, may have the same significance in early right heart failure as a heightened venous pressure. In view, then, of his unaltered ventricular rate, and unaltered venous pressure, it is reasonable to suppose that the decline in ventricular pressures, chiefly systolic, resulted from the transfer of blood from the pulmonary field to the systemic side; and that there was prompt adjustment to this increased systemic arterial input by a lowering of the peripheral resistance. This would account for the absence in this case of the systemic arterial pressure rise seen in the patients with congestive heart failure, in whom later peripheral resistance fall seems to come only after adequate improvement of arterial filling.

The two patients whose cardiac outputs decreased (Nos. 9, 10) were not in heart failure by the usual criteria. Patient No. 10, however, had previously been in failure on several occasions, with heart disease of uncertain etiology. At the time of the present study, both right ventricular systolic and diastolic pressures were slightly elevated; the cardiac index was definitely low. The decline of his ventricular rate from 73 to 60, while not striking, actually represents a stroke volume change only from 49 to 48 cc., so that the decline in cardiac output must here be ascribed almost wholly to the fall in rate, with a failure of ouabain in this case to affect ventricular systole. The slight drop in systemic pressures would be compatible with this decrease in arterial inflow from the left heart.

Patient No. 9 was suffering from rheumatic heart disease with mitral stenosis, not clinically in heart failure; he was able to work as a long-shoreman until admission for study. His only symptom was cough. It will be seen that his cardiac index was also in the lower normal region, and that his ventricular diastolic pressure of 16 mm. Hg was of the order seen in right heart failure. While there was some fall in heart rate between the two outputs, the rates at the time of blood sampling were 81 and 84, respectively. The stroke volume decreased from 60 cc. to 52 cc. The early rise in systemic pressures was not con-

tained, final values being essentially the same as his controls. There was, however, a progressive fall in ventricular diastolic pressure to 8 mm. In view of the nearly 20 per cent increase in peripheral arteriolar resistance, the decline in ventricular filling pressure may simply have represented a diminution of the venous return, as a consequence of decreased arteriolar outflow. The lesion in this instance was not comparable to those present in the other cases, and the action of ouabain, particularly upon a presumably normal left ventricle, was likewise not comparable. Further studies on the effects of ouabain in mitral stenosis are now in progress.

In Patient No. 13, who was in heart failure, the slight variation in all pressures except those in the femoral artery at five minutes after ouabain was consistent with the fact that there was but small variation in the cardiac output and stroke volume. The equal and brief rise in femoral systolic, diastolic and mean pressures five minutes after the drug was given suggests a transitory vasopressor response in this instance. However, the failure to obtain a closer time correspondence between the taking of control pressures and output on the one hand, and the follow-up pressures and outputs on the other, leaves interpretation of this early systemic response at best uncertain. This was the only patient in the series in whom, at the end of the observation period, no clear effect of ouabain upon the circulation was apparent.

The present study confirms in human beings with congestive heart failure the physiological effects of ouabain described in experimental animals as well as those reported in patients by less exact clinical measurements. Ouabain acts directly on the failing heart by increasing its stroke output, and this action may and usually does precede cardiac slowing or any decrease in peripheral venous or right ventricular filling pressure. The latter effects usually do take place, but later. Our results with ouabain are at variance with those reported by McMichael and Sharpey-Shafer⁶ (18) with digoxin, in that they ascribed

the improvement in cardiac output to a fall in right auricular pressure, and in our cases evidence for an improvement in cardiac output preceded any fall in venous pressure. Further studies are now in progress in this laboratory with digoxin as well as other digitalis glycosides.

SUMMARY

1. The intracardiac administration of ouabain in doses of 0.25 to 0.75 mg. to patients in clinical congestive heart failure, suffering from non-valvular types of heart disease, is usually followed by a rise in cardiac output, as seen in nine out of ten patients in heart failure in the present study.

2. Effects on the circulation may be noted within one to eight minutes, and are characterized by an increase in the systemic systolic and pulse pressures and in the right ventricular systolic and pulse pressures. There is a less marked and less uniform early change in the diastolic pressures in these two systems. Where measured, increases in the pulmonary arterial systolic and pulse pressures have also been seen.

3. There is a variability in the later pressure findings, at the end of one to two hours, which may be accounted for by the additional play of other factors such as changes in blood volume and peripheral arteriolar resistance.

4. While there may ultimately be seen evidence of diminished filling pressure in the right ventricle, measurement of the peripheral venous, auricular or ventricular diastolic pressure shows these values to be essentially unchanged at the time of the other pressure rises.

5. The increase in cardiac output is due to an increase in stroke volume.

6. The increased stroke volume can best be explained by a direct action of ouabain upon the contractility of the myocardium.

7. The systemic and right heart pressure changes described are consistent with such an explanation, although vasopressor effects of the drug are not entirely excluded.

8. In three patients the cardiac output was unchanged or fell after ouabain administration. Two of these patients were not in clinical heart failure.

⁶ It should be pointed out that McMichael and Sharpey-Shafer imply peripheral venous pressure alterations, although measuring only central (auricular) pressures. Hemodynamically the two may not be regarded as synonymous.

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THE BILIARY EXCRETION OF BROMSULFALEIN AS A TEST OF LIVER FUNCTION IN A GROUP OF PATIENTS FOLLOWING HEPATITIS OR SERUM JAUNDICE

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Prolonged or permanent impairment of liver function is now known to occur in a variable proportion of patients following an attack of infectious hepatitis or serum jaundice (1-11). However, the incidence of this chronic hepatic disease is not well established because of the difficulty in correlating symptomatology with the available liver function tests or with liver biopsy (12-15).

Of the means available for the study of the post-hepatitis patient liver biopsy has gained favor because the positive findings have a concreteness lacking in the chemical liver function studies. However, it is not without risk to the patient and conceivably early alterations in physiology could precede structural hepatic change by many months (16-20).

The bromsulfalein, cephalin flocculation, thymol turbidity and Van den Bergh are the tests generally employed but frequently the abnormality shown is slight, and is not uniformly present when they are run simultaneously. The blood clearance of bromsulfalein is generally considered a highly useful chemical test when liver impairment is minimal (21). However, we have experimental evidence in dogs which suggests that the clearance of bromsulfalein from the blood is probably dependent upon a dual mechanism; first, removal from the blood by the reticulo-endothelial system (Kupfer cells) and second, excretion by the hepatic polygonal cells into the bile and that early hepatic damage can cause severe impairment of the excretion of bromsulfalein in the bile without interfering with its removal from the blood (22-24). The cephalin flocculation and thymol turbidity tests are not specific tests of liver function because they have been shown to yield positive results in other diseases. Consequently it has been difficult at times to determine the significance of minimal abnormality when it occurs in only one of these tests (25, 26). An elevated serum bilirubin in a post-hepatitis patient is probably a

reliable index of altered liver function but it is not often the case that hyperbilirubinemia exists when the other three tests are normal.

Because of these discrepancies it was felt that it would be of interest to test the excretion of bromsulfalein in the bile in a selected group of patients in whom impaired liver function was suspected but difficult to prove.

MATERIAL AND METHOD

From the hepatitis wards of a military hospital 25 patients were selected because, in spite of continuous treatment for one or two months following an attack of infectious hepatitis (14 patients) or serum jaundice (11 patients) and complete disappearance of icterus of skin and sclera, they were still suspected of having some impairment of liver function.

The patients were divided into four groups on the basis of the following criteria: (1) *prolonged persistence of symptoms* with normal physical findings and normal routine liver function studies; (2) *prolonged abnormal physical findings without accompanying symptoms* and with normal routine liver function studies; (3) *prolonged abnormal routine liver function studies* without symptoms or abnormal physical findings and (4) *prolonged abnormal routine liver function studies and physical findings* without symptoms.

The method of testing liver function by the biliary excretion of bromsulfalein has been described in detail previously and when further reference is made to it in this paper it will be called the biliary bromsulfalein test (22, 23, 27). A duodenal tube is passed and the tip located in the duodenum fluoroscopically. A Lyon or Reh fuss tube may be used but the double-lumened Wallace-Diamond tube is preferred because with suction it is possible to prevent contamination of the duodenal contents with gastric juice. A free flow of bile is established in the usual manner employed in performing duodenal biliary drainage. Bromsulfalein is then injected intravenously (in this study 5 mg. per kilo of body weight was employed) and a blood sample taken at the end of 30 minutes. Bile is collected in 15-minute fractions and the concentration and total quantity of bromsulfalein excreted in the bile over a two-hour period is calculated.

When employing the 5 mg. dose the normal excretion of bromsulfalein presents the following features: (1) 10

per cent, or less, of the dye remains in the blood at the end of 30 minutes; (2) the dye appears usually, but not invariably, in the bile during the first 15 minutes; (3) it attains a maximum concentration in 45 to 75 minutes, falling subsequently to a relatively low level at two hours; (4) approximately 50 per cent of the quantity injected is excreted in the first hour and approximately 75 per cent in the first two hours. Abnormal excretion is evidenced by one or more of the following phenomena: (1) retention of more than 10 per cent of the dye in the blood at the end of 30 minutes; (2) delayed appearance of the dye in the bile; (3) delayed attainment of the maximum concentration; (4) prolonged high curve (plateau-type) of excretion; (5) subnormal concentration (flat type of curve); (6) abnormally low total excretion in one- or two-hour periods after injection (28).

During the initial phase of observation the patient's progress was followed by clinical appraisal and the determination of the icterus index at approximately weekly intervals. When the patient's general condition showed substantial improvement the quantitative and qualitative Van den Bergh, the thymol turbidity test, as modified by Shank and Hoagland (29), and the blood clearance of bromsulfalein were run at approximately weekly intervals. The following values were considered normal: Van den Bergh qualitative, negative direct; quantitative, 0.2 mg. to 0.8 mg. per 100 cc. serum; thymol turbidity, 0 to 4 units;¹ bromsulfalein, 10 per cent or less in the serum at the end of 30 minutes. All determinations were made on the Coleman junior spectrophotometer. The biliary bromsulfalein test was not run until the patient was free of icterus of both skin and sclera, and fell into one of the four categories described above; it was then repeated at intervals of five to 14 days.

¹ At the time this work was done normal values for the thymol turbidity test were based on the work of Shank and Hoagland. Since then, several authors have pointed out that values in excess of 2 units (30) or 2½ units (8) should be construed as abnormal.

RESULTS

Initially all 25 patients showed an abnormal response to the biliary bromsulfalein test. However, after one to two months of additional treatment 60 per cent showed gradual improvement until the response finally approximated normal. While some improvement was noted in the remaining 40 per cent, they continued to show an abnormal response in spite of the one or two months of additional treatment. Unfortunately it was not possible to follow these patients longer so that a final conclusion as to their ultimate improvement could not be drawn. However, the degree of impairment indicated by this test and the slow improvement noted over the three-month period they were observed suggest that some of them suffered a degree of permanent hepatic damage or that a prolonged period of convalescence would still be necessary before complete recovery took place. At the time this study was undertaken circumstances did not permit applying the test to hepatitis convalescents in whom prolonged liver impairment was not suspected. At present, however, we are engaged in following all hepatitis patients on the medical wards with serial testing by this method.

Instead of recording the voluminous data on each patient a typical case has been selected from each of the four categories and a synopsis of his course in the hospital described.

Group I: Prolonged persistence of symptoms with normal physical findings and normal routine liver function studies (four patients).

TABLE I
Tests on patient M. L. (Group I)

Month	Aug.		Sept.				Oct.				Nov.			
Day	24	29	4	11	18	25	3	11	18	23	1	8	12	21
Blood bromsulfalein														
Icterus index	165	265	213	300	160	88			15	18	11	9	10	7.5
Van den Bergh														
Indirect	22	36												
Direct	pos.	pos.					6.5	3.3	1.5	0.7	0.4	0.8	0.9	0.8
Thymol turbidity	9.9						pos.	1.7	1.8	1.3	1.2	1.3	1.4	2.0
*Total dye in bile (%)														
1 hr.										16			13	18
2 hr.										47			49	61
Liver tender	yes	yes	yes	yes	sl.	sl.	sl.	no	no	no	no	no	no	no
Liver palpable	yes	yes	yes	yes	yes	sl.	sl.	no	no	no	no	no	no	no
Weight	195							206	204		206			212

* Biliary bromsulfalein test.

M. L., a 19-year-old infantryman, was wounded in action, sustaining a perforating wound of the right upper arm with transection of the brachial artery. He received plasma on the day of injury and whole blood two weeks later. He was returned to this country and a neurorrhaphy of the right median and ulnar nerves was performed. One month later, the patient noted that his sclera were icteric and his urine dark and a diagnosis of serum jaundice was made. Following a stormy course he appeared to improve objectively but three months following the onset of jaundice he still complained of persistent malaise, headache, inability to concentrate and nausea, belching and fullness after meals.

Although this patient had normal routine liver function studies and revealed no abnormal physi-

cal findings two months after the onset of symptoms (Table I), the initial biliary bromsulfalein test showed considerable impairment of liver function on the basis of poor total excretion of dye as well as an abnormal concentration curve. Approximately one month later there was considerable improvement in the concentration but the total quantity of dye excreted during the first hour was still much less than normal (Figure 1).

Group II: Prolonged abnormal physical findings without symptoms and with normal routine liver function studies (six patients).

G. W., a 25-year-old white infantryman, was admitted with a compound fracture of the left medial malleolus sustained in action on Luzon. He received plasma the day he was injured. Jaundice, anorexia, nausea and dark urine devel-

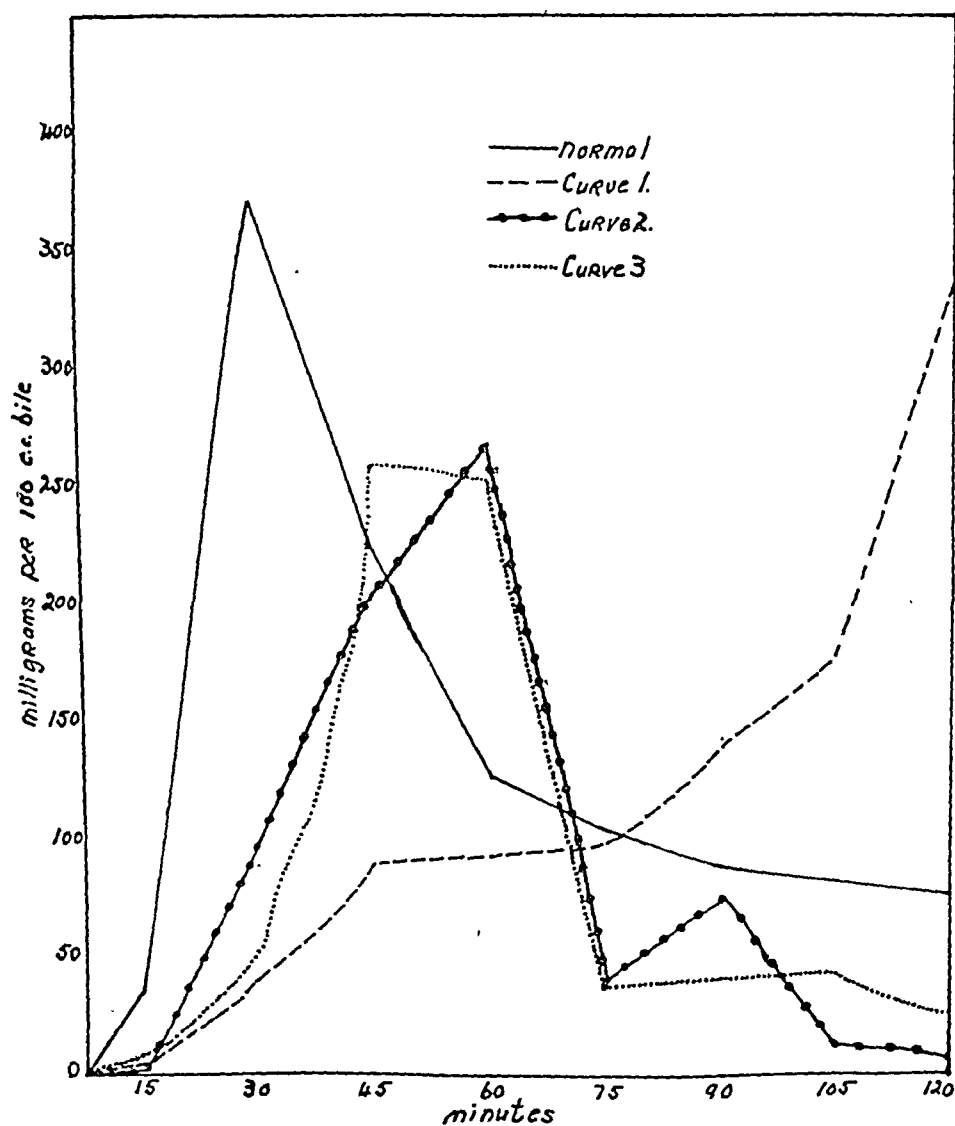


FIG. 1. COMPARISON OF THE BILIARY BROMSULFALEIN CURVES OF PATIENT M. L. WITH NORMAL CURVE

TABLE II
Tests on patient G. W. (Group II)

Month	Aug.	Sept.				Oct.					Nov.		
Day	27	5	11	14	25	3	6	11	18	25	2	8	14
Blood bromsulfalein							10	11	16	5	7	7	5
Icterus index	38	48	56		23								
Van den Bergh													
Indirect			8.0	4.8		0.6	1.2	0.9	0.6	0.6	0.4	0.4	0.3
Direct						—	—	—	—	—	—	—	—
Thymol turbidity							3.4	3.5	3.7	3.7	3.6	3.2	3.4
*Total dye in bile %													
1 hr.											1.2	1	4
2 hr.											5.6	28	40
Liver tender	yes	yes	yes	yes	yes	yes	yes	sl.	sl.	sl.	sl.	sl.	sl.
Liver palpable	yes	yes	yes	yes	yes	yes	yes	sl.	sl.	sl.	sl.	no	no
Spleen palpable	yes	yes	yes	yes	yes	sl.	sl.	sl.	sl.	sl.	sl.	sl.	sl.
Weight	166							176	179	182	184	184	

* Biliary bromsulfalein test.

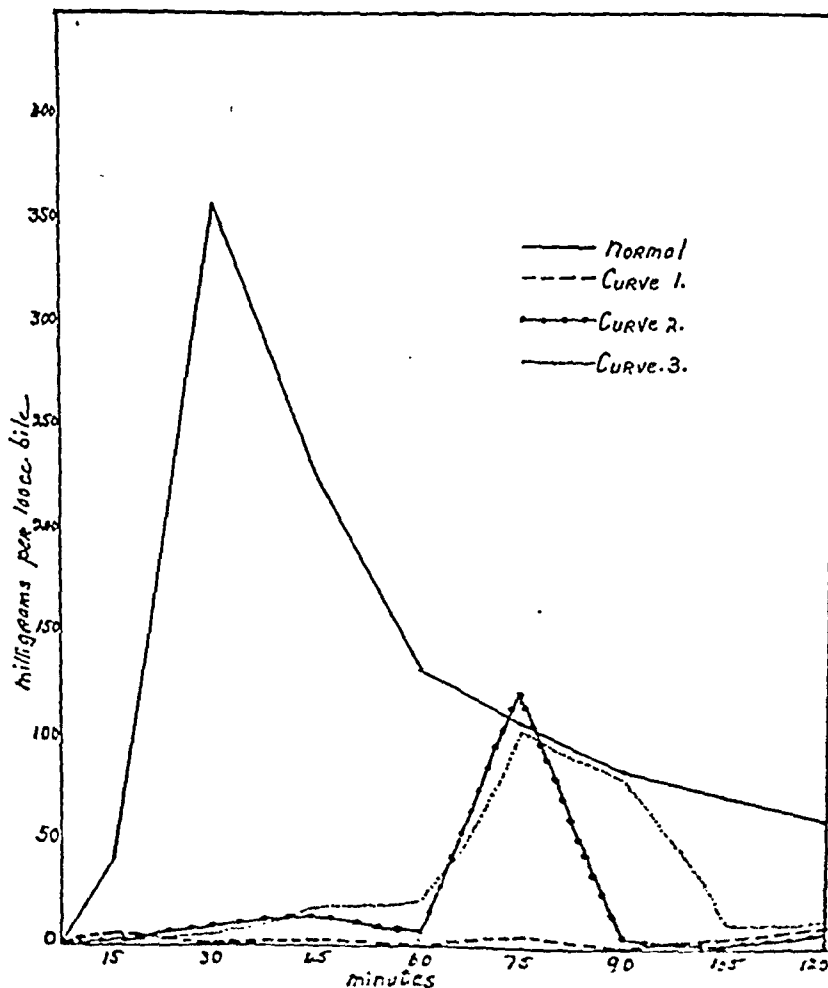


FIG. 2. COMPARISON OF THE BILIARY BROMSULFALEIN CURVES OF PATIENT G. W. WITH NORMAL CURVE

oped five weeks later, while the patient was on the orthopedic ward in a military hospital in this country. Physical examination revealed an enlarged and tender liver and an enlarged spleen. A diagnosis of serum jaundice was made. The patient's convalescence was uneventful except for the persistence of the enlargement and tenderness of the liver and the splenomegaly for a period of over three months.

Although the routine liver function studies were within normal limits (Table II), the biliary bromsulfalein test showed severe impairment of liver function. On the initial test very little of the dye was excreted at all during the test period, so that the concentration curve was almost flat. During the next two weeks there was an increase in the total dye excreted in the two-hour period but the concentration curve remained definitely abnormal (Figure 2).

Group III: Prolonged abnormal routine liver function studies without symptoms or abnormal physical findings (seven patients).

D. M., a 24-year-old paratrooper, was a prisoner-of-war in Germany for six months during which time he developed "trench foot" and lost 30 pounds in weight. While on a hospital train following liberation he developed nausea and vomiting and was noted to be jaundiced. Since he had not received blood or plasma a diagnosis of infectious hepatitis was made. After a month's hospitalization he was symptom-free and was returned to this country. Here physical examination was normal and he was permitted a month's furlough. When he returned liver function studies were performed and found to be abnormal.

Because of this patient's history of infectious hepatitis he was subjected to routine liver function studies before discharge in spite of the fact that he was free of symptoms and abnormal physical findings. These studies revealed an abnormal retention of bromsulfalein in the serum and an abnormal thymol turbidity (Table III). The initial biliary bromsulfalein test, performed after approximately three months' hospitalization, showed moderate impairment of liver function. This improved strikingly over a period of slightly more than a month of continued therapy. The final test was considered within normal limits (Figure 3).

Group IV: Prolonged abnormal liver function studies and physical findings without symptoms (eight patients).

R.K., a 21-year-old paratrooper, was a prisoner-of-war in Germany for four and one-half months, during which time he lost 55 pounds in weight and was in contact with other prisoners who developed jaundice. Two weeks after liberation, the patient developed fever, anorexia, nausea, vomiting, jaundice and pain in the right upper quadrant of the abdomen. A diagnosis of infectious hepatitis was made and the patient was hospitalized for a month and a half before he showed sufficient improvement to permit his return to this country. By this time he had regained 45 pounds in weight and his physical examination was within normal limits. A 30-day furlough was granted but when he returned he was found to have an enlarged liver, which was moderately tender. Liver function study revealed an abnormal thymol turbidity test. The patient improved under further hospital care but the

TABLE III
Tests on patient D. M. (Group III)

Month	July	Aug.			Sept.				Oct.			Nov.				Dec.
Day	3	13	21	29	5	12	14	19	6	23	26	1	8	14	21	5
Blood bromsulfalein	18							16	16	12	11		10	9		5
Icterus index	10	11	23	12	10	13	15	14								
Van den Bergh																
Indirect						0.2	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Direct						—	—	—	—	—	—	—	—	—	—	—
Thymol turbidity	9								9	8				9	6	3
*Total dye in bile (%)	1 hr. 2 hr.										27 43		46 63	41 73		52 61
Liver tender										no		no	no	no	no	no
Liver palpable										no		no	no	no	no	no
Weight	185											194		194		

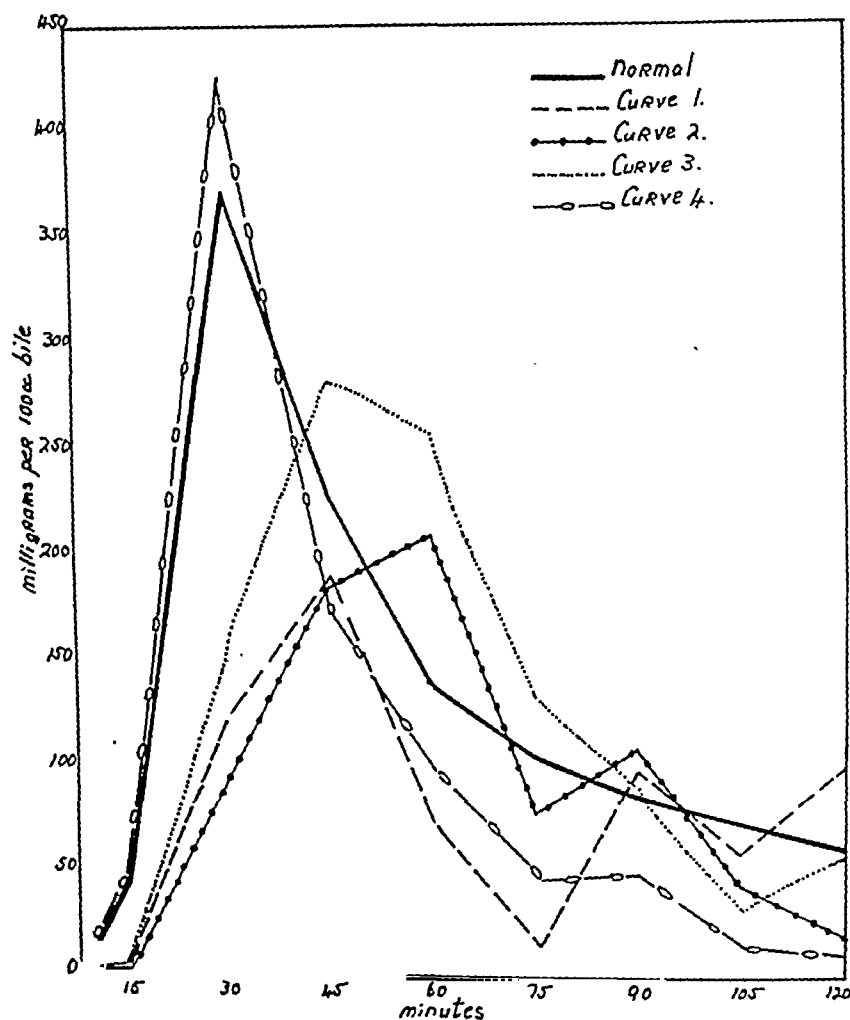


FIG. 3. COMPARISON OF THE BILIARY BROMSULFALEIN CURVES OF PATIENT D. M. WITH NORMAL CURVE

TABLE IV
Tests on patient R. K. (Group IV)

Month	Aug.		Sept.			Oct.						Nov.			
Day	26	31	6	18	25	3	5	11	18	25	29	6	12	21	29
Blood bromsulfalein			3				7	2	3	4	4	3	2	1.5	
Icterus index	13	11	16	16	14										
Van den Bergh			1.1	0.7	1.0	0.4	0.1	1.4	1.0	0.9	1.1	2.5		1.7	
Indirect	1.5														
Direct	pos.		10				7.3	7.8	7.6	7.0	8.5	8.3			4.0
Thymol turbidity															
*Total dye in bile (%)	1 hr.														
	2 hr.														
Liver tender	yes	yes	yes					sl.	sl.	sl.	sl.	sl.	sl.	sl.	sl.
Liver palpable	yes	yes	yes					sl.	sl.	sl.	sl.	sl.	sl.	sl.	sl.
Weight	164							165	167	168	167	167	167	168	

* Biliary bromsulfalein test.

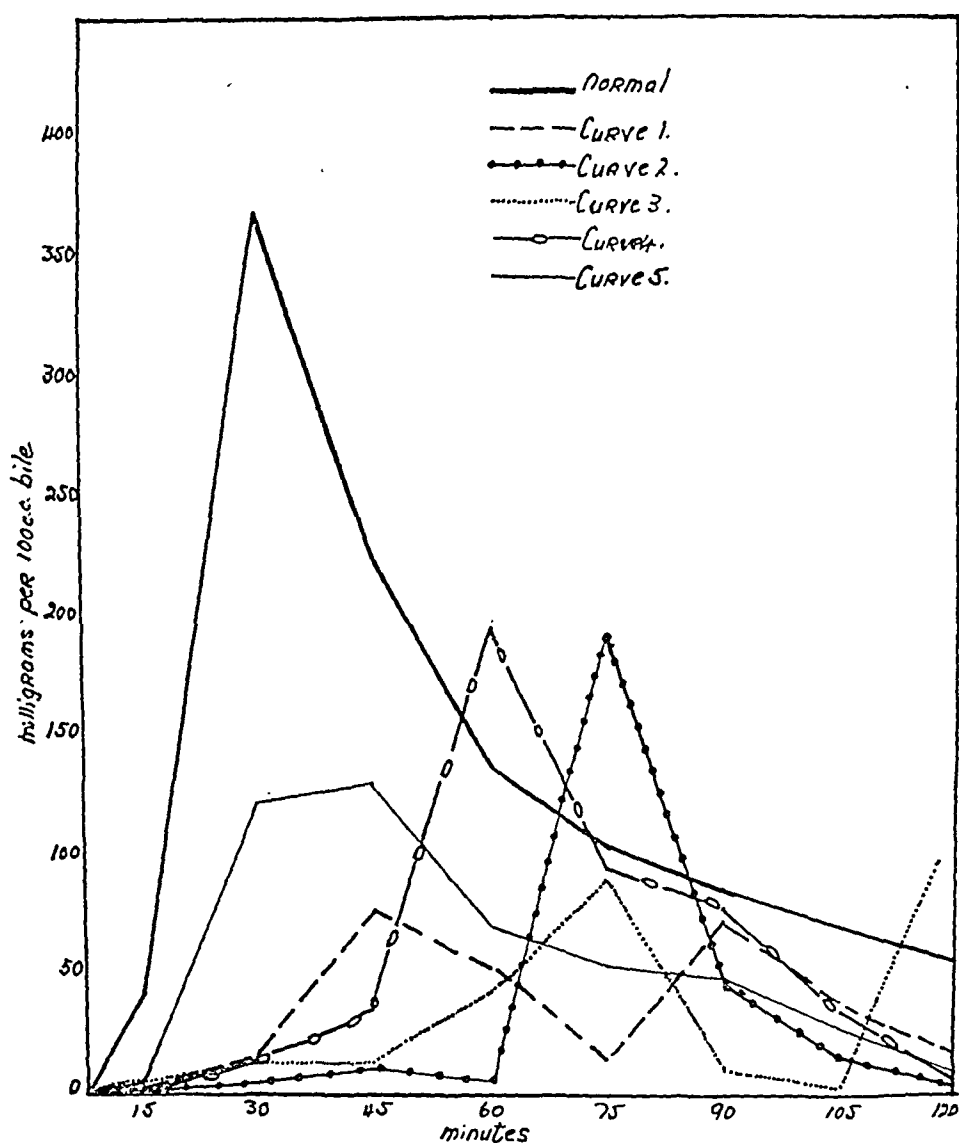


FIG. 4. COMPARISON OF THE BILIARY BROMSULFALIN CURVES OF PATIENT R. K. WITH NORMAL CURVE

liver remained palpable and tender and the thymol turbidity test abnormal for approximately three months (Table IV).

The initial biliary bromsulfalein test, which was performed after the patient had received about two months' treatment, showed severe impairment of hepatic function (Figure 4). Therapy was continued and liver function, according to the biliary bromsulfalein test, improved gradually. The quantity of dye excreted at one and two hours returned to within essentially normal limits over a period of one month, but the concentration curve remained abnormal.

Reference to Table V shows that no significant trend was demonstrated in any of the four groups described above. Actually there was a fairly equal

TABLE V

Group	I	II	III	IV	Total
Number of cases	4	6	7	8	25
Biliary bromsulfalein test normal at 3 months	2	3	5	5	15
Biliary bromsulfalein test abnormal at 3 months	2	3	2	3	10
History of relapse	—	—	4	5	9
Serum jaundice	3	3	2	3	11
Infectious hepatitis	1	3	5	5	14

distribution of those with infectious hepatitis and serum jaundice in whom liver function returned to normal. Those patients who suffered relapse fell exclusively into Groups III and IV, and manifested abnormal routine tests as well as abnormal biliary bromsulfalein tests. However, persistent

abnormal liver function occurred with equal frequency in patients in whom there was no history of relapse.

DISCUSSION

The actual incidence of chronic liver impairment following hepatitis is not well established, and many authors feel that if more sensitive liver function tests were available a higher incidence would be found. Soffer and Paulson (2), using the bilirubin tolerance test, which they considered highly sensitive, found residual impairment of hepatic function in nine of 11 patients, eight of whom had had an acute attack of hepatitis three to 18 years previously. Mateer and his colleagues (21), using a group of newer sensitive tests, found abnormal liver function in 62 patients without clinical or other laboratory evidence of gall-bladder or liver disease. The liver function tests became normal on an average after four months of intensive therapy designed to improve hepatic function.

Kunkel, Labby and Hoagland (10) ran multiple, serial liver function studies at weekly intervals on 350 patients with acute infectious hepatitis. They emphasize the importance of the plasma bilirubin level, the bromsulfalein retention and the thymol turbidity reaction of the serum for following the various types of persistent impairment of the liver. They found eight patients, or 2.3 per cent, who did not recover completely after more than one year's time.

The actual transition from hepatitis to cirrhosis of the liver had been demonstrated by serial biopsy by Iverson and Roholm (16), Dible, McMichael and Sherlock (17), and Hoffbauer (31). Out of 40 patients in whom Mallory performed liver biopsy because of persistence of symptoms following hepatitis, 15 were normal, 10 were doubtful and 15 showed changes similar to a normal convalescent group; that is, histological evidence of periportal and interlobular inflammation and focal hyalin necrosis (32). From these studies it is clear that liver disease can be demonstrated by biopsy when histological change is present, but it seems possible that minimal physiological impairment may not always be manifested by structural change. Similarly it seems possible that minimal structural change could exist in the absence of physiologic abnormality.

In the present study of a group of 25 patients convalescing from hepatitis the results of routine liver function tests were normal or equivocal, but the initial biliary bromsulfalein test showed impaired liver function in all of the patients. By using this test at approximately weekly intervals minimal to moderate impairment of hepatic function was shown to return to normal in 15 patients (60 per cent) when adequate liver therapy was continued long enough. Ten patients (40 per cent) continued to have impaired liver function despite intensive therapy, for at least three months.

CONCLUSION

It appears from this study that the biliary bromsulfalein test is a reliably sensitive test of liver function of considerable help in studying selected patients in whom minimal hepatic functional impairment is suspected.

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STUDIES ON THE MUCOPROTEINS OF HUMAN PLASMA. I. DETERMINATION AND ISOLATION¹

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The presence in plasma of protein-like materials with properties of polypeptides or proteoses has been demonstrated repeatedly, but little has been done toward the characterization of these materials. The concentration of such materials in plasma rises markedly in patients with cancer or with certain other diseases, but here again little is known of the source or significance of the increased levels. The relation of these protein-like materials to the glycoproteins and mucoproteins of plasma is likewise in a state of considerable confusion.

The present series of experiments has grown out of three distinct avenues of attack (a) the demonstration, polarographically, of high molecular weight compounds containing cystine in sulfosalicylic acid filtrates of serum (1 to 6), (b) the demonstration of polypeptide-like substances in serum deproteinized with trichloroacetic acid (7 to 10), and (c) the demonstration of a carbohydrate-rich protein, seromucoid, present in filtrates of serum deproteinized by heat (11 to 14). It appears very likely that these materials are identical, and for reasons that will be developed in this paper, the materials will be referred to as mucoproteins.

Winzler and Burk (6) isolated the non-dialyzable material from sulfosalicylic acid filtrates of rat blood, and compared the properties and occurrence of this material with the older studies of polypeptides. They concluded that the

¹ Suppl product was identical with the substances

Cancer Scted by grants from the National Research Council. Ammittee on Growth, acting for the American providing fiety, and from the National Advisory Cancer

² Some o'e wish to thank the Hancock Foundation for mitted by facilities used in this work.

University these data are taken from a dissertation sub- of the requ. W. Devor to the Graduate School of the phy. Presf Southern California in partial fulfillment

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giving the "index of polypeptidemia" as well as the "polarographic filtrate wave." The material was classed as a proteose because it was soluble in sulfosalicylic acid, was not coagulated with heat, and was salted out with ammonium sulfate. Mayer (15) had previously isolated some of this material from sulfosalicylic acid filtrates of horse serum by precipitating it with alcohol, and called the product a "mucoidähnliche Substanz" because of the high sulfur, carbohydrate and glucosamine contents. Both in the preparations from rat blood and those from horse serum the materials were especially characterized by being very low in nitrogen and high in carbohydrate in comparison to the composition of most proteins. They resembled in this regard the seromucoid isolated from boiled serum by Zannetti (14) and subsequently studied by Bywaters (11), by Hewitt (12) and by Rimington (13).

The present work is concerned with the determination, isolation, and chemical characterization of these materials from normal human plasma, and was undertaken as part of a study of their physiological significance, especially in relation to cancer.

DETERMINATION OF PLASMA MUCOPROTEINS

The concentration of mucoprotein-like substances in plasma has been shown to increase under various pathological conditions, notably in cancer and in pyogenic infections. We have, therefore, investigated chemical methods for the determination of mucoproteins in human plasma, and are reporting our observations in the present communication.

Most of the quantitative work on the plasma mucoproteins in the past has been carried out using plasma deproteinized by means of sulfosalicylic acid or trichloroacetic acid. The effectiveness of these protein precipitants, as well as of perchloric acid (16), has been investigated, the results

showing that addition to human plasma of optimal concentrations of perchloric acid (0.6M) or sulfosalicylic acid (0.2M) leave in the filtrate about 5 mg. of non-dialyzable nitrogen per 100 ml. of plasma, this nitrogen being largely mucoprotein in nature. The most effective level of trichloroacetic acid (0.5M) leaves only about 0.2 mg. of non-dialyzable nitrogen in the filtrate. We have accordingly selected 0.6M perchloric acid as the standard means of separating the plasma proteins from plasma mucoproteins. The protein-like materials in such filtrates have been determined by an adaptation of the method of Burstein (17). This has involved the addition of a strongly acid solution of phosphotungstic acid to the perchloric acid filtrate to precipitate the mucoproteins. The determination of the amount of mucoprotein in the precipitate has been carried out by determining its protein content by means of a quantitative biuret reaction (18), its carbohydrate content using the orcinol reaction (19), its tyrosine content using the phenol reagent of Folin (20), or its nitrogen content using digestion and Nesslerization.

Procedure

To 2 ml. of serum or plasma are added 8 ml. of 0.75M perchloric acid and the mixture shaken. In exactly ten minutes the precipitated proteins are filtered off through Whatman No. 50 filter paper. To 5 ml. of the filtrate is added 1 ml. of 5 per cent phosphotungstic acid in 2 N HCl. In 15 minutes the precipitated mucoproteins are centrifuged down and washed once with phosphotungstic acid.

The tyrosine content³ of the precipitate is determined by dissolving it in 6.5 ml. of $\frac{1}{6}$ saturated sodium carbonate, adding 1 ml. of phenol reagent and, after one hour, reading the color development with a red filter on a Klett-Summerson colorimeter. A standard containing 0.05 mg. of tyrosine is similarly treated.

The carbohydrate content of the precipitate is determined after washing it with 95 per cent ethyl alcohol. The alcohol-washed precipitate is dissolved in 0.5 ml. of saturated sodium carbonate, and 1 ml. of 2 per cent orcinol in 14.3 N H_2SO_4 is added followed by 7.5 ml. of 14.3 N H_2SO_4 . This is mixed well in a large test tube and placed in a water bath at 80° C. for 15 minutes and then cooled in an ice bath. A blank and a standard containing 0.05 mg. of galactose and 0.05 mg. of mannose are similarly treated. The carbohydrate is then deter-

mined from Klett-Summerson colorimeter readings using a green filter.

The protein content of the phosphotungstic acid precipitate is determined by adding 1 ml. of biuret reagent to the precipitate dissolved in 5 ml. of $\frac{1}{6}$ saturated sodium carbonate, and comparing the color with 5 mg. of serum albumin similarly treated using a Klett-Summerson colorimeter and a green filter.

The nitrogen content of the precipitate is determined by digesting it with 1 ml. of 1:1 H_2SO_4 , diluting to 25 ml., and Nesslerizing a 5 ml. aliquot.

Results

Results of the investigation of a number of normal human plasmas and those from a number of patients with cancer are shown in Table I. It is seen that the levels of perchloric acid-soluble, phosphotungstic acid-insoluble tyrosine, carbohydrate, nitrogen and protein are significantly higher than normal in the cancer patients. A more detailed study of the relation of plasma mucoprotein levels to cancer will be the subject of another communication (21).

TABLE I
Plasma mucoprotein levels in normal and cancer patients

	No. of cases	Tyrosine*	Carbohydrate*	Nitrogen*	Protein*	Ratio CHO/T
		mg. %	mg. %	mg. %	mg. %	mg. %
Normal	10	3.38 ± 0.27	12.6 ± 1.1	5.6 ± 0.6	86.7 ± 9.5	3.69 ± 0.23
Cancer†	10	8.53 ± 0.7	33.0 ± 2.6	22.8 ± 1.82	228 ± 15.5	3.85 ± 0.30

* Including the standard error of the mean calculated from the relation

$$SE = \sqrt{d^2/n - 1}/\sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

† The cancer patients were relatively advanced cases and were distributed as follows: 4, carcinoma of cervix with metastases; 3, breast carcinomas with metastases; 2, multiple myeloma; 1, Hodgkins disease.

It is of special interest that in spite of the increased amounts of mucoprotein, the tyro in carbohydrate ratio of about 3.7 is not significantly different from normal in the plasma from patients, and is similar to that isolated from normal human plasma (see Table IV); that the material present in abnormal or cancer blood is similar to that occurring in normal blood. Whether it is identical with and has the same source as that present in normal blood is determined by further experimentation.

One of the factors affecting the determination of plasma mucoproteins by the method

³ Although the values obtained with Folin's phenol reagent are reported as "tyrosine," it is recognized that this reagent is not specific for tyrosine groups in proteins.

TABLE II

Coprecipitation of mucoprotein with perchloric acid-precipitated proteins

Additions in final volume of 10 ml. of 0.6 M perchloric acid	Mucoprotein-tyrosine mg. in 100 ml. P.C.A. filtrate	Recovery of mucoprotein per cent
1 mg. mucoprotein *	3.5	100
140 mg. serum albumin †	0.04	—
1 mg. mucoprotein + 140 mg. serum albumin	2.74	78
1 ml. normal human plasma	3.6	—
1 mg. mucoprotein + 2 ml. normal human plasma	5.9	66

The mucoprotein, serum albumin or plasma or their combinations were made to 6 ml. with distilled water and 4 ml. of 1.5 M perchloric acid were added. Filtration was in ten minutes. Tyrosine determinations were made on 5 ml. of the filtrate as described in the procedure.

* Mucoprotein prepared from perchloric acid filtrates of normal human plasma by $(\text{NH}_4)_2\text{SO}_4$ precipitation and having a tyrosine content of 3.5%.

† Serum albumin prepared by sodium sulfate fractionation.

is the coprecipitation of mucoprotein with the proteins. A study of this coprecipitation was carried out adding to plasma or to serum albumin purified mucoprotein isolated by the method to be described, and determining the recovery in the perchloric acid filtrates. The results of this experiment are shown in Table II. It is seen that the mucoprotein is completely recovered from pure solutions, but with addition of plasma or purified serum albumin the recoveries are markedly reduced.

The apparent mucoprotein levels of plasma are also influenced by the extent of dilution of the plasma before addition of perchloric acid, the levels rising with higher dilution. This is shown in Table III. The increase in plasma mucoprotein levels with increasing dilution is undoubtedly related to the coprecipitation already mentioned.

TABLE III

Apparent plasma mucoprotein levels at different plasma dilution

plasma in 10 ml. of perchloric acid	mg. mucoprotein-tyrosine per 100 ml. plasma*
Some of 0.5	3.8
mitted by A 1.0	3.2
University 1.5	2.8
of the requit 2.0	2.5
phy. Presen 3.0	2.0
College, Min 4.0	1.9
	2.1

nined as described under procedure.

From the experiments shown in Tables II and III, it may be estimated that the values obtained by the methods described are at least 30 per cent lower than those actually existing in the plasma. Attempts to minimize this effect by rapid centrifugation of the perchloric acid-plasma mixture have not been particularly fruitful due to the difficulty of obtaining clear supernatants.

In view of these considerations it is to be expected that the protein level of plasma should have some influence on the apparent mucoprotein level. Since many cancer patients have an associated hypoproteinemia it might be suspected that this could account for the high plasma mucoprotein levels found in cancer patients. However, little correlation has been found between the total plasma protein and mucoprotein levels in normal individuals and cancer patients. Therefore, while the protein content of the plasma is undoubtedly a factor affecting the plasma mucoprotein determination, it cannot account for the difference between normal and pathological bloods.

Because of the ease of determination of the tyrosine content of the mucoproteins we have preferred to carry out the major part of our work with this procedure. It has been also our practice to check the tyrosine results frequently by simultaneous carbohydrate determination since this serves as a means of identifying the phosphotungstic acid precipitate as mucoprotein.

ISOLATION OF MUCOPROTEINS

The most extensive studies on the quantitative occurrence in normal and pathological plasma of the type of material under consideration have been made using filtrates of plasma or serum deproteinized with trichloroacetic or sulfosalicylic acids. Our initial isolations have been therefore made using such protein-free filtrates. Evidence will be presented in other communications showing that these materials are not liberated from proteins under the action of the high acidity of the precipitating reagents, and that they are present in untreated plasma. Isolation of the materials has also been achieved by means of ammonium sulfate fractionation without use of acid precipitants or heat.

Most preparations were isolated from 0.6M perchloric acid filtrates of plasma since it was

found that this concentration left a minimal amount of protein-bound tyrosine and of non-dialyzable nitrogen in the filtrate.

Procedure

To remove the proteins, 500 ml. of plasma were diluted with an equal volume of water, and 500 ml. of 1.8M perchloric acid or of 0.6M sulfosalicylic acid were added while stirring. Filtration through Whatman No. 5 filter paper was started within two to five minutes after protein precipitation. The filtrate was dialyzed nearly free of acid, and the mucoproteins were precipitated by saturating the dialysate with ammonium sulfate at a pH of 4. The materials were exhaustively dialyzed against distilled water and dried by lyophilization. Approximately 20 mg. of the product were isolated per 100 ml. of plasma by this procedure.

Results

The product was a white, fluffy, somewhat hygroscopic material, which dissolved easily in water forming a slightly turbid solution. It gave positive reactions in the usual protein and amino acid tests and also showed a very strong Molisch reaction.

A number of samples isolated by the procedure outlined have been examined using the electrophoretic technique. The results of a typical preparation obtained at pHs of 8.3 and 4.5 are shown in Figure 1. It is seen that the preparation shows at least three electrophoretically demonstrable components. All three of these components have isoelectric points that are much lower than the

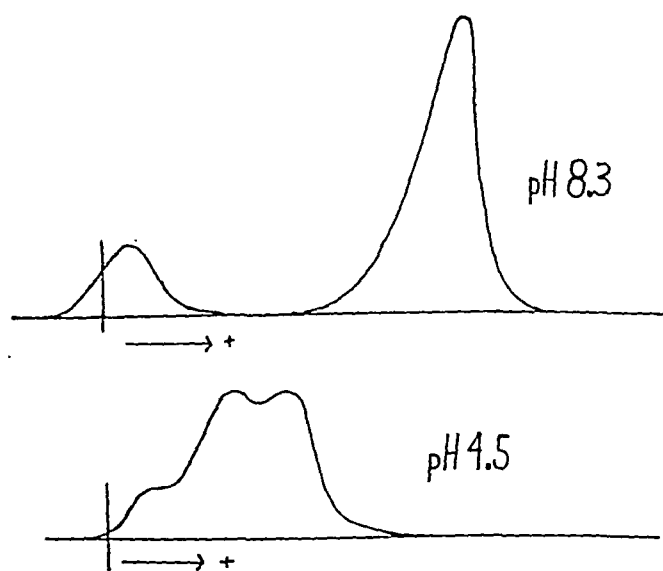


FIG. 1. ELECTROPHORETIC PATTERNS OF MUCOPROTEINS ISOLATED BY THE PERCHLORIC ACID METHOD

plasma proteins. More complete data on the electrophoretic characterization of the mucoproteins will be presented in a later communication. The low isoelectric points observed suggested that with the proper selection of pH and plasma concentrations, mucoproteins might be demonstrable in untreated plasma. This was tried following the suggestion of Dr. M. P. Peterman (22) who has observed in the plasma of patients with gastric cancer a small amount of an electrophoretic component which moved toward the positive electrode at a pH of 4. We have also observed this component at pH 4.5 in a number of patients in whom the plasma mucoprotein levels were elevated. This component has been isolated electrophoretically and characterized chemically, and seems to correspond to one of the plasma mucoproteins observed in our isolated preparations. This problem is under investigation at the present time.

CHEMICAL COMPOSITION

The chemical composition of the materials isolated from pooled normal human plasma insofar as it has been investigated, is tabulated in grams per 100 grams of moisture-free material in Table IV. These studies must be regarded as preliminary because a mixture of components, frequently present in different proportions, is involved. More

TABLE IV
Chemical composition of normal human plasma mucoproteins

Component	Method	gm./100 gm.*†
Ash	Ignition in air	2.8
Nitrogen	Digestion and Nesslerization	7.9
Amino nitrogen (per cent of total N)	Van Slyke nitrous acid method	4.3
Protein	Biuret method (18)	58.0
Carbohydrate	Orcinol reaction (as mannose-galactose) (19)	15.1
Hexosamine	Acetylacetone method (13)	11.9
Hexuronic acid	Carbazole method (23)	neg.
Lipid	Hot alcohol extraction	12.9
Cholesterol	Liebermann-Burchard reaction	neg.
Phosphorus	Molibdivanadate method (24)	0.064
Sulfur	Gravimetric	1.3
Cystine	Polarographic (25)	0.5
Methionine	McCarthy-Sullivan method (26)	2.1
Tyrosine ³	Phenol reagent (20)	4.2
Tryptophane	Erlich reagent (27)	1.8

* Average of ten preparations.

† On moisture-free basis.

detailed chemical studies will await separation of the individual components.

Nitrogen. In these studies, as in those on materials similarly isolated from rat blood and from horse serum, the nitrogen content was very low. Winzler and Burk (6) advanced the hypothesis that the low nitrogen content of their rat blood "proteose" was due to its combination with the sulfosalicylic acid used to remove the plasma proteins. This was based in part on the very high sulfur content of the material. A correction of the nitrogen content to a "sulfosalicylic acid-free" basis brought the nitrogen up to usual protein values. This hypothesis must now be abandoned, since it has been shown that the chemical composition of material isolated from sulfosalicylic acid filtrates and from perchloric acid filtrates is the same, and in neither case indicates the presence of the precipitant. The low nitrogen content of the isolated material must be explained on the basis of its high carbohydrate and hexosamine content as suggested by Mayer (15) for a similar material isolated from horse serum, and in addition on the basis of the high lipid content demonstrated in the present work.

The low nitrogen closely corresponds to the low "protein" content of the material as measured by the quantitative biuret method of Mehl (18). The absorption spectrum of the biuret reaction product given by the mucoprotein shows an absorption peak at 550 $m\mu$, the same as that obtained with a sample of human serum albumin, and suggests a relatively high molecular weight of the mucoproteins.

Seromucoid preparations isolated by various investigators have had nitrogen contents ranging between 7.1 and 13.6 per cent, depending upon the source of the material and the methods of preparation. The nitrogen content of seromucoid preparations have in general varied inversely with their carbohydrate content.

Carbohydrate. The carbohydrate content of the material isolated from human plasma was determined using the orcinol method of Sörensen and Haugaard (19) as well as by a method utilizing the Molisch reaction. Both of these reactions give colors with hexose but not hexosamine, and results with these two methods agreed quite well. Hexosamine was determined by the procedure described by Rimington (13) using acetylacetone

and Erlich's reagent. Tests for hexuronic acids were made by the carbazole method of Dische (23).

It is seen from Table IV that the carbohydrate content is in the neighborhood of 15 per cent based on a galactose-mannose standard. This is somewhat lower than that reported by Mayer (15), but higher than that reported by Winzler and Burk (6) for similar preparations. It lies in the range of the values given for different preparations of seromucoid.

Indication that the carbohydrate is a hexose is afforded by the fact that Tauber's pentose test gave negative results, and that the absorption curve obtained in the Molisch α -naphthol reaction corresponded to that obtained with hexoses rather than to that of pentoses. The color developed in the orcinol test for carbohydrates varies with different carbohydrates, mannose and glucose giving less color than galactose. This makes it essential to know the identity of the carbohydrates determined before accurate determination of the amount present can be made. An indication that the carbohydrate is a mixture of galactose and mannose is afforded by the observation that the ratio of readings obtained in the orcinol reaction at 420 and 540 $m\mu$ with the Klett-Summerson colorimeter corresponded more nearly to a mixture of galactose and mannose than to either alone. Usually this ratio was such as to indicate a relative excess of galactose over mannose. Galactose is known to be present, since mucic acid was isolated from a preparation treated with concentrated nitric acid. The presence of a fermentable sugar was also demonstrated using *Schizosaccharomyces pombe*. These observations are in general accord with those of Seibert *et al.* (28, 29) as well as those of Hewitt (12) and Rimington (13).

In agreement with the observations of Mayer (15) with material isolated from horse serum, of Zanetti (14), Bywaters (11), and Rimington (13) with seromucoid, and of Hewitt (12) with globoglycoid, Table IV shows that our materials have contained considerable amounts of hexosamine. No isolation or identification of this amino sugar from the material isolated from human plasma has been attempted. In view of the identification of glucosamine in horse seromucoid (30), however, it seems likely that the amino sugar present in our preparations is glucosamine.

Hexuronic acids were not present in amounts detectable by the method of Dische (23).

It is clear that the overall analysis of the carbohydrate and hexosamine content of the material isolated in this work places it into the group of mucoproteins according to the classification of Meyer (31), since they contain more than 4 per cent hexosamine. It cannot, of course, be concluded that each component in the mixture contains this same high carbohydrate content. However, in view of the paralleling low isoelectric points, heat stabilities, and solubilities, we have tentatively assumed that all three components are mucoproteins.

In the purest preparations of the seromucoid of Rimington (13), the ratio of hexose to hexosamine was very close to 2:1. Since the mucoprotein prepared in the present work is a mixture of at least three components, an integral ratio is scarcely to be expected. It is of interest, however, that this ratio has usually been much closer to 1:1 than to 2:1 in our preparations. This relation must be reexamined when the components are isolated in pure form.

Lipids. Winzler and Burk (6) reported that lipids were absent in their rat blood "proteose," using ether-extractable material as a criterion of lipid content. Ether has also failed to extract lipids from human plasma mucoprotein. When the mucoprotein isolated from fresh plasma was extracted with hot alcohol in a Soxhlet apparatus, however, considerable alcohol-soluble material was extracted. Preparations isolated from older plasma samples contained considerably less lipid than fresh samples. The white, waxy alcohol-soluble residue was readily soluble in ether or benzene, but not in water. It gave a negative Liebermann-Burchard test for cholesterol, and contained approximately 1.1 per cent nitrogen, 1.1 per cent carbohydrate (orcinol test) and 0.32 per cent phosphorus, the composition varying somewhat in different preparations. All the phosphorus present in the mucoprotein was extracted with hot alcohol.

Sulfur. The sulfur content of the mucoproteins isolated from rat blood (5.8 per cent [6]), from horse serum (3.7 per cent [15]) and from fresh human plasma (1.3 per cent) is greater than can be accounted for on the basis of the sulfur in cystine and methionine. It was found that

after mild acid hydrolysis of the mucoproteins isolated from fresh human plasma, a precipitate could be obtained by treating the hydrolysate with barium chloride, indicating the presence of easily hydrolyzable sulfuric acid. One of the most striking differences in the composition of mucoproteins from the rat, the horse and the human lies in the sulfur content. Whether this represents the result of differences in the methods of preparation or real species differences is as yet not clear.

Studies on the amino acid composition of the mucoprotein are as yet too incomplete to have such significance.

DISCUSSION

The data reported indicate the presence of at least three protein components which have the chemical composition and physicochemical properties that distinguish them from other plasma proteins. The chemical data must be regarded as preliminary since a mixture of proteins has been studied. It is felt that the evidence supports the thesis that all three components are mucoproteins. Evidence to be reported in other communications indicates that the same or similar mucoproteins are present in increased amounts in the plasma of patients with a number of diseases, including many types of cancer, pneumonia, and tuberculosis. That the plasma concentration of similar or perhaps identical materials increases in diseases of different etiology suggests that there is a metabolic abnormality common to all. Studies on the source and physiological significance of the plasma mucoproteins should be of considerable value in testing this hypothesis.

There can be little doubt that the total serum polysaccharides studied by Seibert and her collaborators (28, 29) include the mucoproteins described here. Their total serum polysaccharide levels lay in the neighborhood of 100 mg. per cent in the normal individual, this value rising to as high as 150 or more in patients with cancer or tuberculosis. The normal plasma mucoprotein level expressed in terms of carbohydrate is 14.4 ± 3.2 mg. per cent and may rise to 75 mg. per cent in cancer patients. Whether or not the increase in total serum polysaccharide in pathological blood observed by Seibert is entirely due to increase in plasma mucoproteins cannot yet be ascertained, although the similarity in the absolute increase in

carbohydrate suggests that this may be the case.

It is unlikely that the conjugated amino acids studied in tungstic acid filtrates of plasma by Christiansen and Lynch (32) have any relation to the mucoproteins studied here, since the mucoproteins are precipitated by this reagent. There is some question as to whether the conjugated amino acids in plasma deproteinized with 2.5 per cent trichloroacetic acid contain mucoproteins, since we have found that this concentration of trichloroacetic acid is just under that required for optimal precipitation of plasma proteins, and that it leaves in solution small amounts of relatively high carbohydrate-containing proteins.

SUMMARY

Methods for the determination of the mucoprotein content of normal and pathological human plasma have been developed. Plasma from cancer patients contains higher than normal amounts of mucoprotein.

A mixture of mucoproteins has been isolated by saturating perchloric acid filtrates of normal human plasma with ammonium sulfate at pH 4. Electrophoretic studies indicate the presence of three components all with low isoelectric points. The material is high in carbohydrate, hexosamine and sulfur, and low in nitrogen content in comparison to other proteins.

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STUDIES ON THE MUCOPROTEINS OF HUMAN PLASMA. II. PLASMA MUCOPROTEIN LEVELS IN CANCER PATIENTS¹

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A number of studies have indicated that there exist in blood, protein-like materials which are not readily precipitated by heat, by perchloric acid, by sulfosalicylic acid, or by high concentrations of trichloroacetic acid. The concentration of such materials has been found to increase in the blood of patients afflicted with cancer as well as in patients with a number of other diseases.

In a program designed to elucidate the nature of this material and its possible significance in cancer, we have isolated the material from normal human plasma and have shown it to be a mixture of mucoproteins (1). Improved methods for the determination of plasma mucoproteins have also been studied (1).

This paper is concerned with an investigation into the plasma mucoprotein level in two conditions in which the concentrations have been found to be increased over the normal levels.

EXPERIMENTAL

Most of the determinations were carried out using the determination of perchloric acid soluble, phosphotungstic acid-insoluble tyrosine² of plasma as previously described (1). In certain experiments the carbohydrate content of this same fraction was determined in order to ascertain whether the carbohydrate-tyrosine ratio deviated significantly from normal in pathological sera.

Results on the determination of mucoprotein-tyrosine levels in the plasma of 337 normal individuals and 454 cancer patients are shown in Figure 1. The normals were largely taken from negative premarital Wassermann sera. All of the cancer cases included in Figure 1 have been diagnosed with certainty by biopsy or at autopsy. The disease in most cases was relatively far advanced. The extent of the work at the present time does not justify the grouping of the data as to

type or extent of the malignancy. The results shown in Figure 1 show that the normal plasma mucoprotein levels are in the range of 1 to 4 mg. of tyrosine content per 100 ml. plasma and averaging 2.7 ± 0.05 mg.%, whereas the serum from cancer patients ranges between 2 and 12 mg.% averaging 6.1 ± 0.13 mg.%. There is considerable overlapping between the high normals and the low cancers, but the maximum frequencies are distinctly different in the two groups.

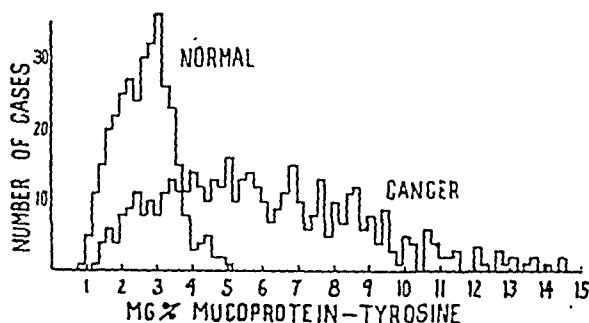


FIG. 1. PLASMA MUCOPROTEIN LEVELS IN NORMAL INDIVIDUALS AND IN CANCER PATIENTS

These results are in general accord with the work of previous investigators using polarographic determination of sulfosalicylic acid filtrates of denatured serum as well as with studies on the "index of polypeptidemia" (2 to 10).

Figure 2 shows the plasma mucoprotein level as a function of time in three pneumonia patients admitted to the hospital. It is seen that these patients had very high plasma mucoprotein levels initially when the body temperatures were at a maximum. These high levels fell to normal levels and paralleled the temperature and the recovery of the patient.

The observation that patients suffering from pyogenic infections have increased amounts of "protein split products" in the serum is in accord with the results of other investigators listed above. Crossley and his associates (11) have made an extensive investigation of changes in the

¹ This work was supported by a grant from the National Research Council Committee on Growth, acting for the American Cancer Society.

² Although the results are reported as "tyrosine" it is recognized that the Folin phenol reagent is not specific for tyrosine groups in proteins.

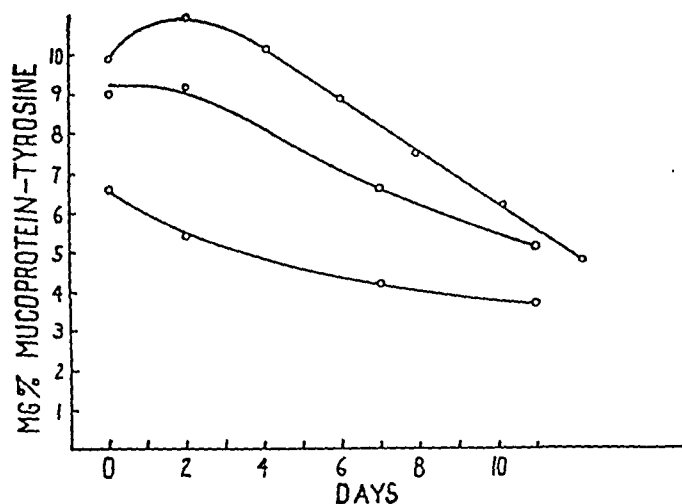


FIG. 2. THE PLASMA MUCOPROTEIN LEVELS OF PATIENTS WITH BRONCHIAL PNEUMONIA

plasma proteins including "peptone," of experimental pneumonia in dogs. Their work also shows a parallel relationship between serum "peptones" and the development of the pneumonia and subsequent recovery. In a recent paper, Vassel *et al.* (12) have isolated "proteose" from normal dog plasma and from that of dogs with pneumonia by fractionation with ammonium sulfate. They obtained increased amounts of this material in the infected dogs and showed that it contained about 25 per cent carbohydrate (expressed as mannose, galactose and glucosamine).

Although the method employed determines primarily mucoproteins in normal individuals, the possibility exists that the type of material determined may be altered in pathological states. This question is being investigated by direct isolation from larger quantities of blood. However, some

indication as to the similarity of the material in normal plasma and from the patients with cancer or pneumonia is given in Table I which shows the tyrosine and carbohydrate content of the perchloric acid-soluble, phosphotungstic acid-insoluble material in plasma determined as previously described (1). The results show that the carbohydrate/tyrosine ratios of the materials in plasma which are soluble in 0.6 M perchloric acid but are precipitated by phosphotungstic acid, are constant in spite of the quite marked deviations in absolute levels of tyrosine. Thus it may be concluded that the materials responsible for the increased mucoprotein levels in pneumonia and cancer (and presumably other conditions) are chemically similar to the mucoprotein that has been previously isolated from normal human plasma (1).

DISCUSSION

The data presented in this paper show that the plasma mucoproteins are increased over normal levels in a large percentage of patients with cancer. However, the increased levels are not constant or specific enough to be of great value as a diagnostic test for cancer. False positive tests are given in pneumonia and a number of other conditions. Likewise, false negative tests are given in many cancer patients, especially when the disease is not far advanced. However, levels of over 4 mg. of mucoprotein-tyrosine per 100 ml. of plasma are rarely found except under pathological conditions. A carefully controlled study of the possible use of the method for diagnosis of cancer of the upper gastrointestinal tract is currently in progress. The very fact that the plasma mucoproteins are elevated in diseases of such different etiology as cancer, pneumonia and myocardial infarctions (Simkin *et al.*, 13), suggests that some abnormality in protein metabolism may be common to all of these conditions. It will be of utmost importance to investigate the chemical composition and physicochemical properties of mucoprotein isolated from pathological plasma in order to determine whether they are similar to or different from those present in normal plasma. It will also be important to investigate the source of the plasma mucoproteins and to determine their physiological significance.

TABLE I

Tyrosine and carbohydrate ratios of mucoproteins in normal and pathological blood

	Number of cases	Tyrosine*	Carbohydrate*	CHO* T
		mg. %	mg. %	
Normal	19	3.0 ± 0.2	11.2 ± 1.0	3.70 ± 0.21
Cancer†	22	6.7 ± 0.9	24.5 ± 2.8	3.66 ± 0.17
Pneumonia‡	15	7.2 ± 0.8	26.9 ± 2.2	3.74 ± 0.20

* Including the standard error of the mean calculated from the relation

$$SE = \sqrt{d^2/n - 1/\sqrt{n}}$$

where "d" is the deviation from the mean and "n" is the number of observations.

† Relatively advanced cases.

‡ Blood taken from patients with lobar or bronchial pneumonia while the fever was at its height.

SUMMARY

An investigation has been made of the plasma mucoprotein levels in 337 normal individuals and 454 patients with cancer. The normal plasma mucoprotein-tyrosine levels averaged 2.7 ± 0.05 mg.% while the levels in cancer patients averaged 6.1 ± 0.13 mg.%. Patients with pneumonia also showed markedly increased plasma mucoprotein levels. The ratio of carbohydrate to tyrosine was about 3.7 in the plasma mucoprotein in both of these conditions as well as in the normals, suggesting the similarity of the plasma mucoproteins in all cases.

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TABLE II

Subject	Oxygen consumption	Metabolic rate—devia. from normal basal	Oxygen satur. of arterial blood	Oxygen content of arterial blood	Arterial-mixed venous oxygen diff.	Arterial-hepatic venous oxygen diff.	Cardiac index	Hepatic blood flow	Hepatic blood flow as percent. of cardiac output	Splanchnic oxygen consump.	Splanchnic oxygen consump. as percent. of total oxygen consump.
	ml. per min. per sq. M.	per cent	per cent	vol. per cent	vol. per cent	vol. per cent	liters per min. per sq. M.	ml. per min. per sq. M.	per cent	ml. per min. per sq. M.	per cent
J. G.	166	+33	88	12.0	6.1	8.8	2.7	460	17	41	25
L. B.	185	+44	90	19.6	8.4	12.8	2.2	200	9	26	14
J. S.	155	+25	97	19.1	10.2	11.0	1.5				
B. S.	160	+36	89	16.9	8.2	9.1	2.0	530	27	48	30
J. R.	206	+56	92	16.7	7.7	9.6	2.7	680	25	65	32
T. J.	161	+22	89	16.1	7.7	9.2	2.1	370	18	34	21
L. S.	124	+ 2	92	9.6	5.9	5.9	2.1	800	38	47	38
J. D.	117	-13	89	16.0	8.0	8.8	1.5	420	29	37	32
E. W.	162	+34	96	14.9	6.5	7.7	2.5	650	26	50	31
F. B.	110	-14	96	18.0	7.2	8.0	1.5	390	25	31	29
J. A.	140	+ 9	96	17.0	5.3	6.2	2.6	540	21	33	24
C. T.	148	+19	97	19.7	5.5	5.5	2.7	680	25	37	25
E. B.	131	+ 7	92	17.6	6.0	6.6	2.2	700	32	46	35
Cardiacs: mean	151	20			7.1	8.4	2.2	535	24	41	28
st. dev.*	27	21			1.4	2.0	0.5	170	7	11	7
S. E.†	7.5	5.8			0.4	0.6	0.14	49	2.0	3.2	2.0
Controls: mean	153	13			3.9	4.5	4.1	850	20	38	24
st. dev.*	16	10			0.5	0.8	0.5	170	4	10	8
S. E.†	5.3	3.3			0.2	0.2	0.17	45	1.3	2.9	2.7

* standard deviation.

† standard error.

the estimation of surface area. However, only two patients (L. S. and J. D.) had edema or effusions of such degree as might significantly affect calculations.

As compared to normal individuals (2, 3), relatively higher concentrations of bromsulphalein were obtained in cardiacs from a given dose of the dye per minute. The 13 patients obtained a mean concentration in arterial serum of 2.55 ± 1.79 mgm.⁴ per 100 ml. from an average infusion rate of 2.5 ± 0.97 mgm. per min. The 12 control subjects showed a mean arterial concentration of 1.61 ± 0.75 mgm. per 100 ml. from an average infusion rate of 5.82 ± 1.47 mgm. per min. The percentage extraction of bromsulphalein ($[\text{arterial serum concentration} - \text{hepatic venous serum concentration}] \div [\text{arterial serum concentration}]$) varied widely but in general was again subnormal in the patients with heart failure, they

averaging 26 per cent extraction as compared to 50 per cent for the controls. As the percentage extraction varies inversely with the arterial concentration, the control subjects with their low average arterial concentration of bromsulphalein would be expected to have a higher percentage extraction. However, it seems that the difference in arterial concentration does not account for the difference in extraction between the controls and the cardiacs. From our data, the control subject at an arterial concentration of 2.55 mgm. per 100 ml. (the average arterial concentration for the group with heart failure) would have a predicted extraction of 37 per cent. This is to be compared with the 26 per cent extraction found in the heart failure group at that average arterial concentration.

The data on the removal of bromsulphalein from the liver of heart failure, in comparison with the normal liver, are better expressed in terms of bromsulphalein clearance ($[\text{total removal rate BSP in mgm. per min. per sq. M.}] \div [\text{concentration of BSP in mgm. per ml. of arterial serum}] = \text{ml. of arterial serum cleared completely}$

⁴ Throughout this paper in the expression of \pm values, the standard deviation, s , is used, where

$$s = \sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{n}}{n-1}}$$

BSP per min. per sq. M.). In our series of 14 subjects without significant disease the bromsulphalein clearance ranged from 152 to 365 ml. per minute per sq. M. with a mean of 233 ± 71 ml. The cardiac patients gave clearance of from 30 to 149 ml. per min. per sq. M. for a mean of 74 ± 45 ml.

It has been shown (2) that bromsulphalein in the blood stream is protein-bound and that dye will be lost into the urine in the presence of proteinuria. Only three of the present series of cardiac patients (B. S., J. R., and J. D.) had proteinuria by the usual qualitative tests at the time of the measurement of hepatic blood flows. These proteinurias were all of moderate degree and it does not seem that the amount of dye lost into the urine would seriously affect the determinations.

Oxygen consumptions in the group with cardiac failure ranged from 110 to 206 ml. per min. per sq. M. of body surface. The corresponding metabolic rates varied between minus 13 and plus 56 per cent of the normal basal with a mean of plus 20 per cent. In this respect the patients with cardiac failure did not differ significantly from the controls who had a mean metabolic rate under the conditions of this study of plus 13 per cent of the normal basal.

None of the patients demonstrated a marked degree of unsaturation of the arterial blood for oxygen. The patients with more severe degrees of heart failure, however, did have moderate unsaturation.

The values for hematocrit and oxygen content of arterial blood in Tables I and II show that only two patients (J. G. and L. S.) had significant anemia. It is unlikely that the degree of anemia in subject J. G. played any great part in altering his circulatory measurements, as Brannon *et al.* (6) have found that depression of the hemoglobin to below 7 g. per 100 ml. is necessary before one gets a significant effect on total cardiac output. In the case of L. S., however, it cannot be assumed that her anemia may not have affected the data. It is noted that L. S. had the highest hepatic blood flow of any patient with heart failure. It has been observed (7) that severe degrees of anemia in the absence of heart failure are associated with elevated hepatic blood flows, which

in general are proportionate to the elevation in total cardiac outputs due to the anemia.

All patients with cardiac failure had elevated arterial-mixed venous oxygen differences, the values ranging from 5.3 to 10.2 volumes per cent and the mean being 7.1 ± 1.4 volumes per cent. The control subjects by comparison gave differences of from 3.3 to 4.7 volumes per cent with a mean of 3.9 ± 0.5 volumes per cent. Arterial-hepatic venous oxygen differences corresponded, the cardiacs showing elevated values varying from 5.5 to 12.8 volumes per cent for a mean of 8.4 ± 2.0 volumes per cent. The control group had differences of from 3.8 to 5.8 volumes per cent with a mean of 4.5 ± 0.8 volumes per cent. Thus all the patients with cardiac failure, with the exception of one (C. T.) who had only mild failure, showed decidedly elevated arterial-hepatic venous differences. In the cardiacs, as in normal persons (3), the arterial-hepatic venous difference in general exceeds the arterial-mixed venous difference by a small amount.

Every patient with heart failure had a subnormal cardiac index (cardiac output in liters per min. per sq. M. of body surface), particularly when it is remembered that the nature and duration of the circulatory measurements reported here tend to increase the circulation somewhat above the basal. The control group had cardiac indices between 3.3 and 4.9, the patients with heart failure indices from 1.5 to 2.7. The means of these determinations are for the controls 4.1 ± 0.5 , and for the cardiacs 2.2 ± 0.5 .

The estimated hepatic blood flows vary over a considerable range in both the control and heart failure groups. This is probably explainable, at least in part, when it is remembered that the flow through only one portion of the liver is being determined, and that the flows as estimated from several areas of the same liver under the same conditions may show considerable variation (2). Furthermore, several of the patients in the heart failure group had low extraction percentages for bromsulphalein. Under this circumstance, the usual technical errors in measuring the dye concentrations in arterial and hepatic venous bloods have a considerably greater influence on the calculated hepatic blood flow. The hepatic blood flows for the patients with heart failure ranged from 200 to 800 ml. per min. per sq. M. of body

surface; the controls gave values of from 600 to 1160 ml. per min. per sq. M. The average values for the cardiacs are 535 ± 170 ml. and for the controls 850 ± 170 ml. This overall difference is statistically significant ($p < 0.01$). When the values for cardiac output and hepatic blood flow in each patient are correlated, it is found that, in both the control group and the series with heart failure, the hepatic blood flow represents a fairly constant percentage of the cardiac output. Among the cardiacs the hepatic blood flow made up on the average 24 ± 7 per cent of the cardiac output; in the controls it comprised 20 ± 4 per cent. Three patients with heart failure show considerable disproportion between the two values. Patient L. B., who had severe heart failure at the time of study, had a very low hepatic blood flow and at the same time an extreme hepatic arterio-venous oxygen difference. He is the only patient in our experience to manifest a much more marked reduction in liver blood flow than in cardiac output. Whether this does occur with any frequency in severe heart failure can only be told by the study of more patients. Patient L. S., with an arterial oxygen content of only 9.6 volumes per cent, had a high liver blood flow in proportion to her cardiac output. One can only conjecture concerning the effect of her considerable anemia in explaining this discrepancy. Patient E. B. also had an hepatic blood flow which was proportionately large for her cardiac output. E. B. had a chronically enlarged liver, and palpable spleen and, as can be seen in Table I, a marked retention of bromsulphalein, 7.69 mg. per 100 ml. of arterial serum from a moderate dose of 3.51 mg. of dye per minute. At her extraction percentage of bromsulphalein of only 8 per cent, the measurement of liver blood flow is subject to considerable error. Thus the figure of 700 ml. per min. per sq. M. is open to question. It seems likely that E. B. had chronic liver disease out of proportion to that usually encountered in congestive heart failure; possibly this liver disease may have locally affected hepatic blood flow (8). In only one other patient in the series, (J. D.), were there clinical indications of the existence of liver disease which was out of proportion to the degree and duration of the cardiac decompensation.

The splanchnic oxygen consumption (hepatic blood flow \times hepatic A-V oxygen difference) in

the patients with heart failure ranged from 26 to 65 ml. per min. per sq. M. as compared with the control range of 26 to 53 ml. The corresponding mean values are, for the cardiacs, 41 ± 11 ml. and, for the controls, 38 ± 10 ml. When the splanchnic oxygen consumptions for the two groups are expressed as percentages of the respective total oxygen consumptions, again essentially identical values are obtained, 28 ± 7 per cent for the cardiacs and 24 ± 8 per cent for the controls.

DISCUSSION

Before discussing what implications can be drawn from the above data, several precautions must be sounded in connection with the interpretation of such data. Firstly, circulatory measurements as have been reported in this paper have such inherent variations as to show quite wide discrepancies even among similar individuals (9). Secondly, any measurements made by the procedure of catheterization of one of several hepatic veins are measurements by sampling and again considerable variation among similar subjects is to be expected. Lastly, our studies by their very nature could not be performed under consistently basal conditions, the element of anxiety to the procedure being particularly variable. It must be stressed therefore, that comparison of any one case with another might well lead to erroneous conclusions, and that only groups of cases should be compared. Thus the mean values reported here are the pertinent figures.

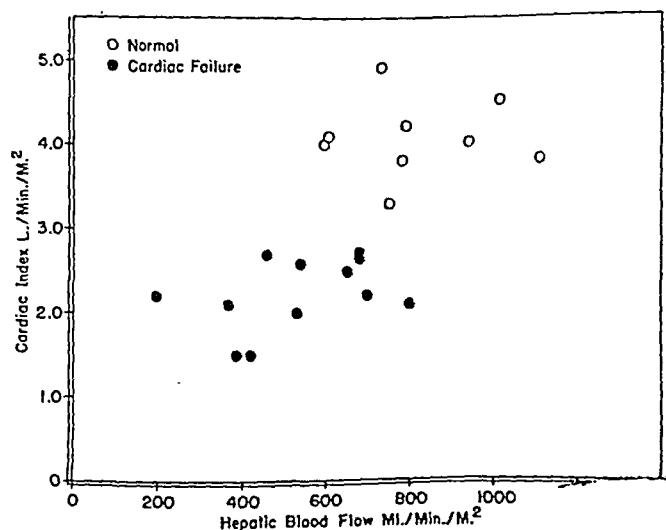


FIG. 1. THE RELATIONSHIP OF HEPATIC BLOOD FLOW TO CARDIAC OUTPUT IN CONTROL SUBJECTS AND 1 WITH HEART FAILURE

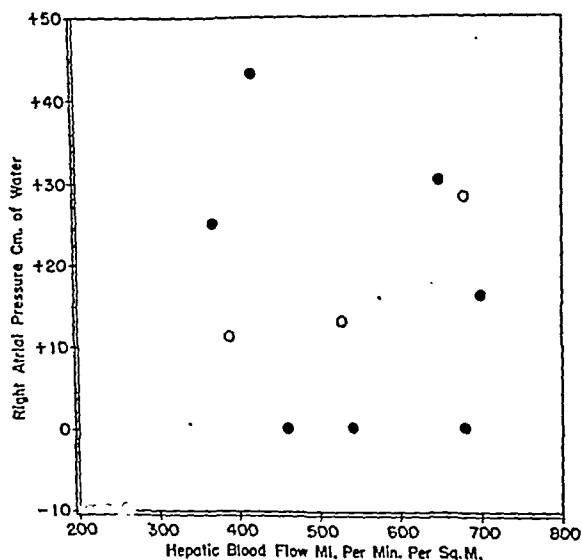


FIG. 2. HEPATIC BLOOD FLOW AT VARIOUS LEVELS OF RIGHT ATRIAL PRESSURES (BLACK DOTS) AND PERIPHERAL VENOUS PRESSURE (OPEN CIRCLES)

The above data indicate that in cardiac failure there is a decrease in hepatic blood flow, and that this decrease is roughly proportionate to the decrease in total cardiac output (Figure 1). Mechanisms by which this decrease in flow might be brought about are (a) a decreased inflow of blood due to a decrease in total cardiac output, (b) vasoconstriction of the afferent vessels to the liver, similar to that presumed for the kidney in heart failure (4), (c) an increased resistance in the hepatic capillary bed as from edema or other pathologic changes in the liver itself, and (d) impairment to outflow of blood from the liver because of an elevation of hepatic venous pressure as a part of the general elevation of venous pressure found in heart failure. The finding that the mean hepatic blood flow in heart failure expressed as a percentage of the total cardiac output, 24 ± 7 per cent, is the same as for the control subjects, 20 ± 4 per cent, is of particular significance in that it indicates that there is no selective reduction of the liver blood flow. Thus possibilities (b) and (c) above are eliminated as accounting, by themselves at least, for the reduced flow. Examination of Tables I and II and Figure 2 shows that there is poor correlation between the level of hepatic blood flow and the corresponding right atrial or antecubital venous pressure. For example, patient E. W. had an hepatic blood flow within the lower

normal range, 650 ml. per min. per sq. M., in the presence of a right atrial pressure of 30 cm. of water. Several patients do show reduced hepatic blood flows in conjunction with elevated pressures. However, both phenomena may be parallel manifestations of severe heart failure and are not necessarily directly interrelated. It seems, then, that the liver blood flow in heart failure is governed, in large part at least, by the level of the cardiac output. The liver receives its usual percentage of the total output, in this respect differing strikingly from the kidney which undergoes a marked and disproportionate reduction in blood flow.

It has been considered that the so-called central necrosis of liver lobules found in heart failure is a manifestation of an increased hepatic venous pressure. The finding of similar lesions in severe anemia and anoxemia (10) without elevation of venous pressure throws doubt on the above correlation. Our finding of a marked unsaturation of hepatic venous blood in cardiac failure is in agreement with the hypothesis that central necrosis may be related to a low oxygen concentration in the blood bathing those liver cells which lie farthest from the afferent blood supply.

The individual in heart failure with a reduced cardiac output maintains a normal body oxygen consumption by an increased extraction of oxygen from that blood supplied to the various viscera, that is, by an increased arterial-mixed venous oxygen difference. This is demonstrated for our

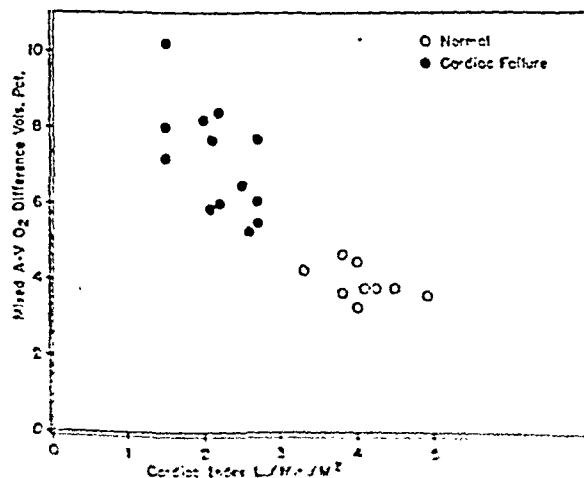


FIG. 3. RELATIONSHIP OF MIXED ARTERIO-VEINOUS OXYGEN DIFFERENCE TO THE CARDIAC OUTPUT IN CONTROL SUBJECTS AND PATIENTS WITH HEART FAILURE

group of patients with cardiac failure in Figure 3. The moderate reduction in blood supply which the liver suffers in heart failure is accompanied by a similar compensatory increase in extraction of oxygen from that blood supplied to it (Figure 4). This increase in arterial-hepatic venous oxygen difference allows the body to maintain, *under conditions of rest and fasting*, a normal splanchnic oxygen consumption. It seems reasonable, under the conditions of our study, that the splanchnic oxygen consumption, which is the oxygen

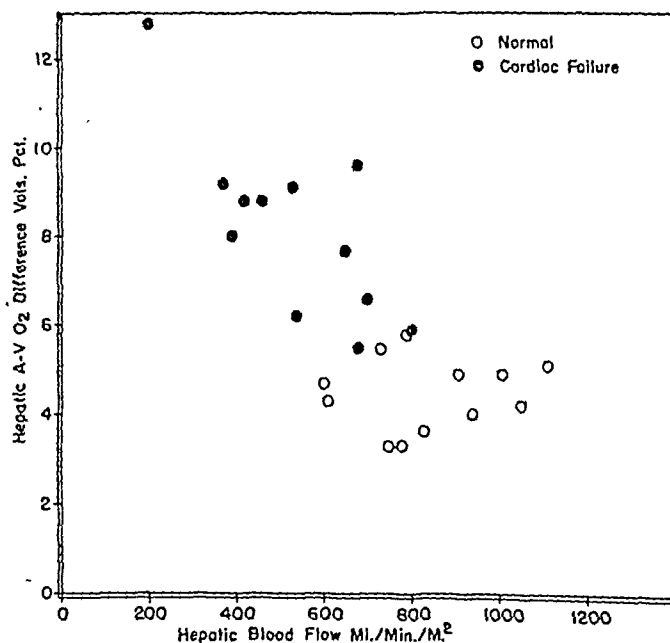


FIG. 4. RELATIONSHIP OF HEPATIC ARTERIO-VENOUS OXYGEN DIFFERENCE TO HEPATIC BLOOD FLOW IN CONTROL SUBJECTS AND PATIENTS WITH CARDIAC FAILURE

consumption of the gut, pancreas and spleen as well as the liver, is reflective of the true hepatic oxygen consumption. The finding of a normal splanchnic oxygen consumption, then, is in agreement with the clinical observations that the liver of heart failure maintains most of its function quite well, *e.g.*, the formation of plasma proteins. One awaits with interest, however, studies as to the effects of various stresses, such as exercise and the intake of foodstuffs, on the liver blood flow and oxygen consumption. Several of our patients (*e.g.*, J. S., J. B.), with cardiac failure had such large arterial-hepatic venous oxygen differences that little further increase in splanchnic oxygen consumption could be accomplished without an increase in blood flow. Since many in-

dividuals with severe heart failure are incapable of an increase in cardiac output so as to be able to augment hepatic blood flow (11), an increase in the latter could only be provided, if at all, by a diversion of blood from some other portion of the body. The liver of heart failure, then, when under stress, might well find its supply of oxygen and other metabolites inadequate to meet normal metabolic demands.

SUMMARY

1. The hepatic blood flow in a group of 13 patients with cardiac failure, as estimated by the bromsulphalein method, varied from 200 to 800 ml. per min. per sq. M. of body surface with a mean of 535 ± 170 ml. The hepatic blood flows in a group of 14 controls studied under similar circumstances ranged between 600 to 1160 ml. for a mean of 850 ± 170 ml. Statistical analysis shows the difference between the means of the two groups to be highly significant.

2. The mean cardiac indices for the two groups were 2.2 for the cardiacs and 4.1 for the controls. The hepatic blood flows expressed as percentages of the total cardiac outputs averaged 24 per cent for the cardiacs and 20 per cent for the controls.

3. The arterial-hepatic venous oxygen differences for the patients with heart failure varied from 5.5 to 12.8 volumes per cent, as compared to a range of 3.3 to 5.8 volumes per cent in the controls. Mean values were 8.4 volumes per cent for the cardiacs and 4.5 volumes per cent for the controls.

4. The average splanchnic oxygen consumption in heart failure was 41 ml. and for the controls 38 ml. per min. per sq. M. of body surface. These values represent 28 per cent and 24 per cent, respectively, of the total oxygen consumption.

CONCLUSIONS

1. The hepatic blood flow in cardiac failure is moderately reduced, and in proportion to the reduction in total cardiac output.

2. This reduction in liver blood flow is compensated by an increase in arterial-hepatic venous oxygen difference so as to provide, under conditions of rest and fasting, a normal splanchnic oxygen consumption.

ACKNOWLEDGMENT

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THE EVALUATION OF AN EFFECTIVE DOSAGE OF CARONAMIDE (4-CARBOXYPHENYLMETHANESULFONANILIDE) FOR THE SUPPRESSION OF TUBULAR EXCRETION OF PENICILLIN IN CHILDREN^{1, 2, 3, 4}

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It has been shown by Beyer *et al.* in a recent series of papers (1 to 4) that the compound 4-carboxyphenylmethanesulfonamide, known as Caronamide, is effective in increasing the penicillin plasma concentration and in prolonging the period of time over which detectable penicillin plasma concentrations are maintained following administration of penicillin, orally and by venoclysis, to dogs. In these experiments it was demonstrated that the renal tubular excretion of penicillin could be completely suppressed (2) when the antibiotic was given concomitantly with adequate amounts of Caronamide.

In a series of experiments in children (5), Caronamide used in conjunction with penicillin administered orally was effective in increasing the penicillin plasma concentration from 2.8- to 14.5-fold over the penicillin controls and was also found to be of a low order of toxicity at the dosages employed.

The purpose of the present research was to determine an optimum dose of Caronamide for children.

METHODS

Method of administration. Afebrile convalescent patients, aged two to nine years, with normal renal function were selected. Single doses of approximately 300,000 units of penicillin/sq. M. of body surface (equivalent to approximately 10,000 units/Kg. of body weight) were given intramuscularly as a control, and blood was drawn

for assay at intervals of one-half,⁵ one, three, five and seven hours after administration. The penicillin used was the regular crystalline commercial variety containing 90 per cent penicillin G. One of the authors always made the dilutions (with physiologic saline) and administered the dose. At intervals of two days thereafter, the same patients received the same dose of penicillin intramuscularly together with Caronamide orally, the Caronamide dosage being increased on each successive trial. The daily dose of Caronamide ranged from 5 to 20 Gm./sq. M./24 hours. The daily doses of 5, 10, and 15 Gm./sq. M. were divided into six equal aliquots given at four-hour intervals. Two aliquots were given as priming doses before, and the third simultaneously with the intramuscular dose of penicillin. The largest daily dose, 20 Gm./sq. M., was divided into eight equal aliquots given at three-hour intervals with three aliquots given as priming doses, and the fourth with the penicillin. This different interval was used in order to reduce the size of individual aliquots at this high dosage level.

Method of assay. A modified Rammelkamp assay technique (6) was employed, using half-step dilutions of the test plasma with a hemolytic streptococcus⁶ as the test organism.

RESULTS

The object of this study was to determine how great a dose of Caronamide is required to suppress completely the tubular excretion of penicillin, reducing it to that of glomerular filtration alone. Since the measurement of direct clearance values was impracticable, the results were obtained by indirect means.

Eagle and Newman (7) have shown that the renal clearance of penicillin approximates the total renal plasma flow and is four to five times greater than the glomerular filtration alone. In other words, 20 per cent of the penicillin is excreted by

¹ This study was supported by a grant-in-aid from Sharp & Dohme, Inc.

² The Caronamide used in this study was supplied by Dr. Karl Beyer of the Research Department of Sharp & Dohme, Inc.

³ The Sharp & Dohme proprietary name for this compound is now "Staticin" Caronamide.

⁴ Some of the penicillin used in this study was kindly supplied by Commercial Solvents Company, Ltd.

⁵ Time at which it was assumed that complete equilibrium between plasma and extracellular fluid had been achieved.

⁶ Kindly supplied by Drs. Verwey and Miller, of Sharp & Dohme, Inc.

the glomeruli and 80 per cent by the tubules. As the tubular excretion is decreased the proportion of the penicillin excreted by glomerular filtration is increased, the actual rate of renal clearance of penicillin being proportionately diminished, until 100 per cent of the penicillin is excreted by glomerular filtration, and the rate of plasma clearance becomes constant at approximately one-fifth of the initial rate. In these experiments, the slopes obtained by plotting the levels of penicillin concentration measured were considered as indicating renal clearance of penicillin.⁷ Since the rate of plasma penicillin clearance follows an exponential curve the rates of clearance at different dosages of Caronamide could be compared to the rate of clearance of penicillin alone by comparing the straight line slopes of the decreasing plasma penicillin levels as plotted on semi-logarithmic paper. The minimum dose of Caronamide resulting in a

A.A. 9yrs. Wt. 26.36Kg. Surface Area 1.02 sq.m.

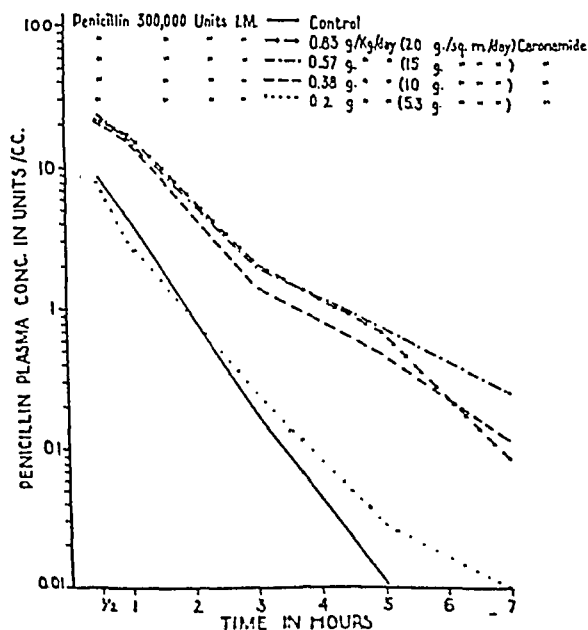


FIG. 1. PATIENT A. A. CHARACTERISTIC PENICILLIN EXCRETION CURVES WITH DIFFERENT DOSES OF CARONAMIDE

⁷ The question of disappearance of penicillin by means of inactivation by the binding properties of plasma proteins does not materially modify this assumption in the case of penicillin G. Tompsett, Shultz and McDermott (14) have shown that 84 per cent of injected penicillin G is excreted in the urine.

J.S. 6yrs. Wt. 18.4 Kg. Surface Area 0.74 sq.m.

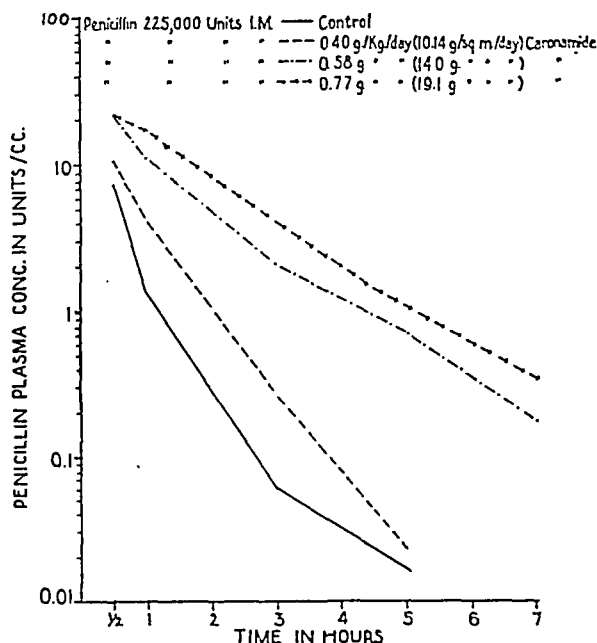


FIG. 2. PATIENT J. S. CHARACTERISTIC PENICILLIN EXCRETION CURVES WITH DIFFERENT DOSES OF CARONAMIDE

maximum decrease of excretion rate was taken as the dose causing complete suppression of tubular excretion (see Figures 1 and 2). For the purpose of convenience, these clearance rates are given numerical values as calculated from a standard formula,⁸ and are referred to as constants of excretion rates (K). (See Tables I and II.)

⁸ The formulae used for calculating (1) the slope of the decrease of the measured plasma penicillin levels and (2) the slope of the calculated decrease of penicillin levels expected with glomerular filtration alone, are given below.

In both, K = the constant rate of clearance of the extracellular fluid of penicillin by the kidney in a given unit of time.

Formula 1:

The standard formula (15) for the calculation of the rate of decrease of the plasma concentration of a substance by means of a unimolecular reaction is $-\frac{dc}{dt} = Kc$. Here c = the concentration of penicillin, K = proportionately factor or constant, and $-\frac{dc}{dt}$ = rate at which the penicillin concentration decreases. By integration this becomes

$$K = \frac{\log_2 C_1 - \log_2 C_2}{t_2 - t_1}$$

or

$$K(t_2 - t_1) = \log_2 \frac{C_1}{C_2}$$

TABLE I
Patient A. A.

Grams of Caronamide/ Kg./24 hours	Constants of excretion rates (K)
0. (control)	1.61
0.2	1.18
0.38	0.94
0.57	0.82
0.83	0.85
Estimate complete suppression	0.82

A second indirect approach was also used. It has been shown by Newman *et al.* (8) that there is a direct mathematical relationship between the rate of decrease of the plasma concentration of a substance, the rate of glomerular filtration, and the volume of the extracellular fluid compartment. Using this relationship it was possible to calculate the rate of decrease of plasma penicillin concentration that might be expected if only glomerular filtration were occurring. The calculation was made according to a standard formula⁸ in which the normal average glomerular filtration rate was

$$\begin{aligned} \text{Let } t &= \text{time in hours,} \\ \text{and let } t_2 - t_1 &= 1. \\ \text{Then } K &= \log_e \frac{C_1}{C_2}. \end{aligned}$$

Converting to common log for direct comparison with the plotted graphs

$$K = \log \frac{C_1}{C_2} \times 2.3$$

(2.303 = factor for converting natural logarithm to common logarithm)

Formula 2:

The slope of the curve (K) which would exist if penicillin were excreted by glomerular filtration alone can be calculated from the following formula derived from the mathematical relationship existing between plasma concentration, extracellular fluid volume and the rate of glomerular filtration in cc./min. (8).

$K = \frac{R}{V}$ where R is the glomerular filtration rate and V is the volume of extracellular fluid.

Since the plasma concentrations measured in Formula 1 were taken at hourly intervals, R was taken to equal 120 (average cc./min.) \times 60.

$V = 20$ per cent of body weight in kilograms, expressed as cubic centimeters.

Since the average glomerular filtration rate was derived from adults with an average surface area of 1.7 meters, the filtration rate of each child was converted to the same basis by multiplying R by the area in square meters of the child (A) divided by 1.7.

The final formula then was:

$$K = \frac{120 \times 60}{20 \text{ per cent body wt. in Kg. as cc.}} \times \frac{A}{1.7}$$

TABLE II
Patient J. S.

Grams of Caronamide/ Kg./24 hours	Constants of excretion rates (K)
0. (control)	1.58
0.4	1.41
0.58	0.75
0.77	0.74
Estimate complete suppression	0.85

used (120 cc./min.) and the extracellular fluid volume was assumed to be 20 per cent of the body weight. In this way, a theoretical constant (K) for the rate of plasma penicillin glomerular clearance was obtained for each child. This was used as a standard with which the constant of the slowest rate obtained with Caronamide in the same child was compared. Complete identity of the measured slope with, or a rate slower than, the estimated standard slope was taken as 100 per cent tubular suppression; faster rates were expressed as percentage of tubular suppression attained by that dose of Caronamide.

The graphic representation of the excretion slopes with Caronamide also demonstrates the delayed excretion rate in terms of the time of disappearance of penicillin from the blood stream. In two of three cases, levels of over 0.02 units/cc. were still present at 12 hours.

As previously mentioned, the Caronamide dosage was based on the body surface area as a unit of measurement because this is known to be more closely correlated with renal function than is body weight. Since, however, this is an uncommon unit in clinical practice, it was thought advisable to recalculate the doses in terms of body weight.

Figure 3 is a graphic summary of the findings. At a dose of 0.2 Gm./Kg./24 hours, there is no appreciable suppression except in one case. At approximately 0.4 Gm./Kg./24 hours, there is a wide scattering of effect from 23 to 100 per cent, with an average suppression of 56.4 per cent. Between 0.55 and 0.70 Gm./Kg./24 hours, there is suppression of from 71 to 100 per cent, with an average of 91 per cent. Fifty per cent of these trials are estimated as showing total suppression. Between 0.70 and 0.92 Gm./Kg./24 hours, the average suppression is 97 per cent. From these data, 0.55 to 0.70 Gm. of Caronamide/Kg./24 hours appears to represent an effective dosage range of this drug in the age-group studied. On

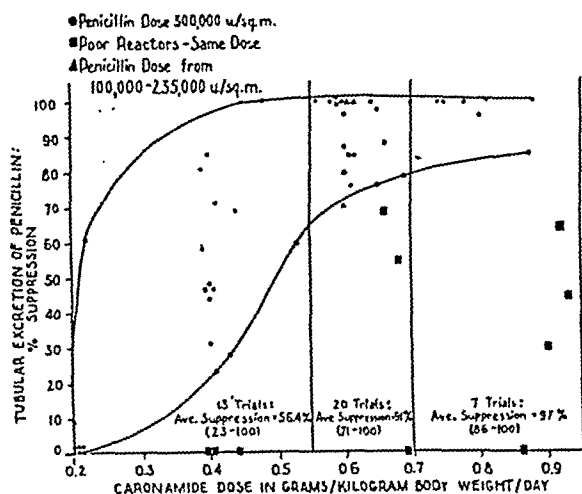


FIG. 3. TUBULAR EXCRETION OF A FIXED DOSE OF PENICILLIN WITH INCREASING DOSES OF CARONAMIDE PER KG. OF BODY WEIGHT

The lines represent the upper and lower limits of the percentage suppression obtained with a Penicillin dosage of 300,000 U/sq. M.

the basis of surface area, the younger children in the age-groups studied will probably require the larger doses per Kg. within this range, whereas

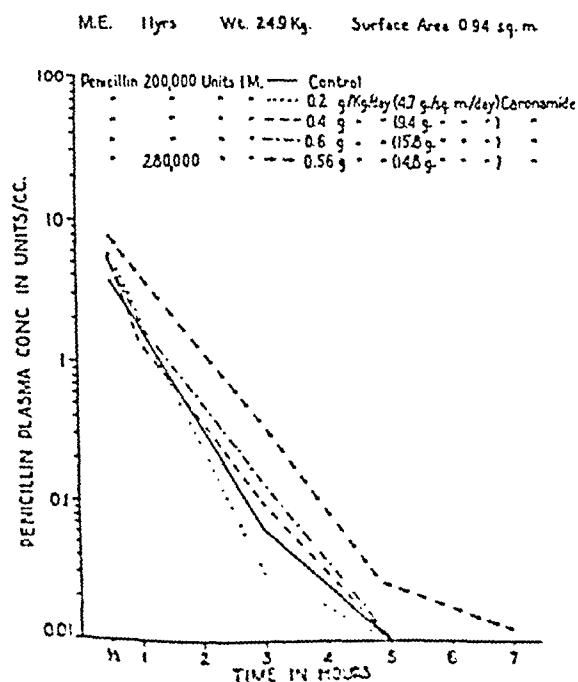


FIG. 4. PATIENT M. E. PENICILLIN EXCRETION CURVES CONSISTENTLY FAIL TO SHOW MORE THAN A MINIMAL DECREASE IN RATE EVEN WITH CARONAMIDE DOSES WITHIN THE NORMALLY EFFECTIVE RANGE

older children, above the weight of 27 Kg., will probably need progressively smaller doses per Kg. within the range. In still older children, it would be reasonable to use the adult dosage schedule recommended by Boger (9), of a total of 24 Gm./24 hours, in six or eight divided doses.

Effective tubular suppression was not obtained in some children, even with Caronamide doses higher than those in this range. One of these is illustrated in Figure 4. A few individuals, such as the one shown in Figure 5, responded incon-

P.A. 4yrs. Wt. 13.98 Kg. Surface Area 0.61 sq. m.

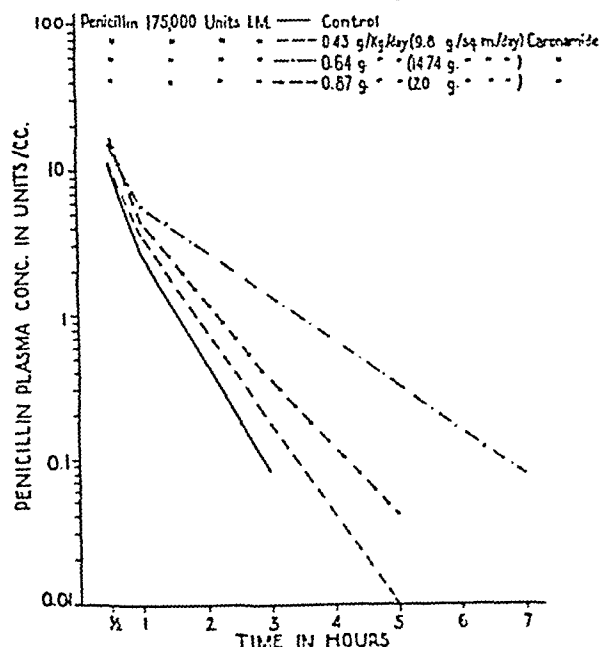


FIG. 5. PATIENT P. A. PENICILLIN EXCRETION CURVES SHOW AN INCONSISTENT RESPONSE TO DOSES OF CARONAMIDE IN THE NORMALLY EFFECTIVE RANGE

The response to the largest dose is less than to a smaller dose.

sistently; in this instance a dose of 0.8 Gm./Kg./24 hours produced less effect than a lower dose of 0.6 Gm./Kg./24 hours, which caused complete suppression. In patient M. E., illustrated by Figure 4, only a minimal improvement over the control was obtained in four trials at varying doses up to 15 Gm./sq. M. These failures are apparently due to variability of Caronamide absorption. This was substantiated by failing in patient M. E. a urinary excretion of only 0.25 per cent of the ingested Caronamide over a 24-hour per-

riod, whereas a consistently good reactor (patient A. A., Figure 1) showed a 24-hour excretion of 60 per cent and 67 per cent of ingested Caronamide, respectively, in two trials. In the poor reactor (patient M. E.) no Caronamide was demonstrated in the plasma in two out of three trials; a plasma concentration of less than 5 mg. per cent on the third trial was present. In adults, Boger (9) has shown that a plasma Caronamide concentration of 20 to 40 mg. per cent was required to give satisfactory blocking of the tubular excretion of penicillin. Of a total of 23 trials (15 patients) at doses within the recommended range, 0.55 to 0.70 Gm./Kg./24 hours, significant tubular suppression was obtained in 20 trials (12 patients), with no response or inadequate response in three (three patients). Thus, a satisfactory response was obtained in 87 per cent of the trials.

Figure 6 illustrates that with an effective dose of Caronamide the rate of penicillin excretion, at three different dosage levels, is the same. The levels reached by 100,000 units with Caronamide are rather better than those reached by three times the dose without Caronamide. Figure 7 shows that with an effective dose of Caronamide,

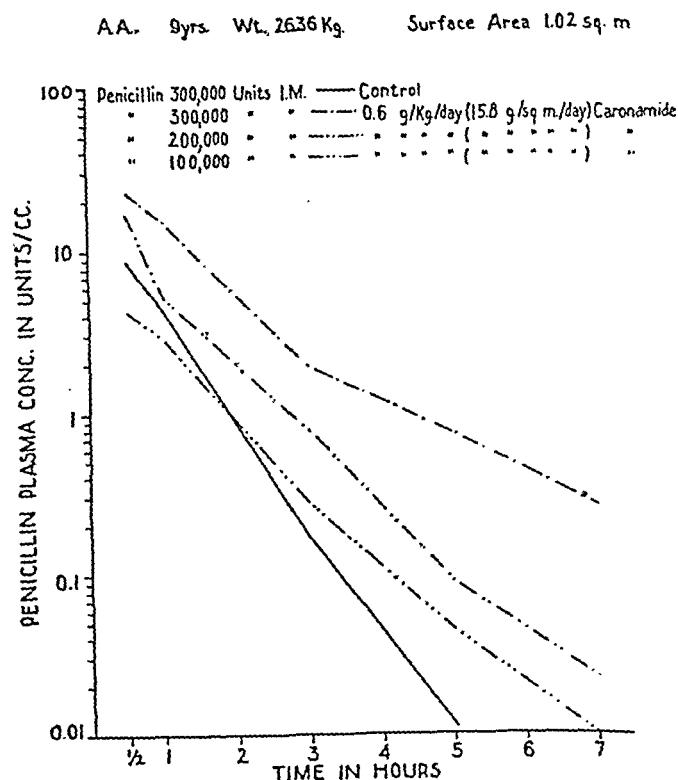


FIG. 6. PATIENT A. A. COMPARISON OF PENICILLIN EXCRETION CURVES OF THREE DIFFERENT PENICILLIN DOSES USING AN OPTIMAL DOSE OF CARONAMIDE

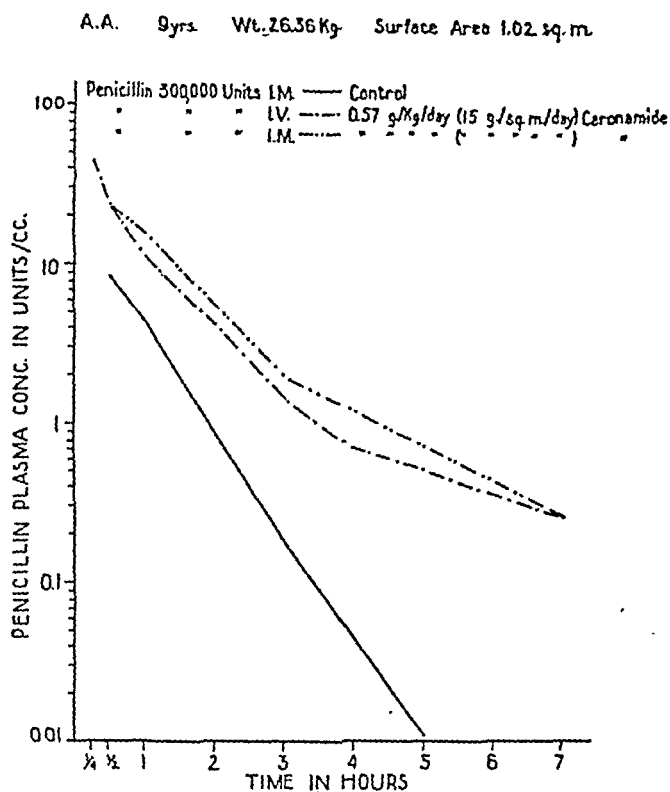


FIG. 7. PATIENT A. A. COMPARISON OF THE CURVES OF PENICILLIN EXCRETION AFTER INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION USING AN OPTIMAL DOSE OF CARONAMIDE

the excretion of penicillin given intravenously is at the same rate as that given intramuscularly. An identical result was obtained in three different patients.

Toxicity

In this study, toxicity of Caronamide was found to be of a very low order, confirming the impression obtained in earlier work (5). Toxic reactions were encountered on only five occasions out of the 59 tests conducted on 21 patients. In four patients, these consisted of anorexia, nausea and vomiting, and then only at doses higher than the dose range now considered advisable. In three of these children, there was no reaction with high daily doses given at four-hour intervals, but the same daily dose given at six-hour intervals with correspondingly larger individual doses produced nausea and vomiting. In the fifth patient a transient macular itching rash appeared over the face, neck, and shoulder girdle after nine doses of Caronamide. This began to fade four hours after discontinuing the drug and had disappeared within 36 hours.

It seems important to record here two severe sensitivity reactions observed after this series was completed: One was a 2½-year-old white boy, R. J. (chart No. 46579), who had a seborrheic dermatitis associated with marked sensitivity to the staphylococcus aureus. He was given one course of penicillin and Caronamide lasting 16 days without apparent reaction. Six days later, he was started on a second course of Caronamide at a dosage of as low as 0.27 Gm./Kg./24 hours. After two doses of 1.25 Gm. each, he developed an extensive maculo-papular rash with a rectal temperature of 103° F. A white count done at the height of this reaction showed a slight polymorphonuclear leucocytosis. The reaction faded within 24 hours of stopping medication. A test dose given five days later led to a similar but more severe reaction. The second was a white boy of three years, D. T. (chart No. 46387), being treated for subacute bacterial endocarditis. He was given Caronamide (0.6 Gm./Kg./24 hours) for two days without reaction. Six days later, he was given one dose of 1.5 Gm. Four hours later, he developed rigors, a temperature of 105° F. and a diffuse erythema. All these signs subsided in 12 hours without therapy.

DISCUSSION

The data presented suggest that in children of the age range studied, two to nine years, complete or almost complete suppression of the tubular excretion of penicillin can be achieved in over 85 per cent by the oral administration of optimal doses of Caronamide. The result of such a suppression is to raise the plasma concentration of penicillin at any given dose and to prolong the time during which such a level is maintained. It would appear that in clinical medicine this fact would be valuable in a situation where the patient is infected with a comparatively resistant organism, since very high blood levels can be attained and maintained by suitably adjusting the size and frequency of penicillin administration in conjunction with the recommended dosage of Caronamide. This treatment has been used effectively for resistant cases of subacute bacterial endocarditis (10, 11). It also may be of value in reducing the need for frequent doses of penicillin by prolonging the maintenance of a theoretically effective blood level for organ-

isms of all degrees of sensitivity. A single dose of penicillin per 24 hours may be sufficient for very sensitive organisms. The results of Zubrod's work (12) suggest, however, that infrequent but large doses of penicillin may be effective therapeutically without the addition of Caronamide. It is not within the scope of this paper to discuss the best clinical application of these data since this must be determined by actual clinical trial. It may well be that smaller doses of Caronamide, at longer intervals, giving less than complete tubular suppression will be entirely adequate for the treatment of most infections. On the other hand, it seems more probable that the need for Caronamide may be confined to those situations just mentioned, in which very high and prolonged blood levels are needed for the therapy of infections by resistant organisms. With the recent development of a method for determining blood levels of Caronamide, a closer correlation between therapeutic effect and blood levels will be possible. Using this method, Boger (9) has found that there is a tendency for Caronamide to accumulate in the blood so that the dosage schedules may have to be adjusted according to blood levels. Also some method may be found of improving the absorption of Caronamide from the intestinal tract and thus make the drug valuable to the 15 per cent of patients who now fail, temporarily or permanently, to respond. In this study, no attempt has been made to evaluate the dosage for infants under two years of age. The recognized inefficiency of the immature kidneys of infants up to six months at least (13) would suggest that smaller doses of Caronamide would be needed in this age-group to effect the same results on the penicillin excretion rate.

SUMMARY

A comparison of the excretion rates of penicillin given intramuscularly alone and with varying oral doses of Caronamide was used to determine the dose of Caronamide effecting complete suppression of tubular excretion.

By this method, an estimated 71 to 100 per cent, with an average of 91 per cent, suppression was achieved in 20 trials with doses of Caronamide between 0.55 and 0.70 Gm./Kg./24 hours in children between two and nine years.

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By this method, an estimated 71 to 100 per cent, with an average of 91 per cent, suppression was achieved in 20 trials with doses of Caronamide between 0.55 and 0.70 Gm./Kg./24 hours in children between two and nine years.

Of a total of 23 trials at doses within this range, such a suppression of tubular function failed to occur in three. This was apparently due to a failure of absorption of Caronamide from the gastrointestinal tract.

Suppression of tubular excretion led to the maintenance of effective blood levels of penicillin for as long as six to eight hours after the administration of a dose of approximately 10,000 units/Kg.

Toxic symptoms of anorexia, nausea, and vomiting were encountered in four patients only with doses higher than those finally recommended. One mild drug rash was encountered in this series. Two other children, not in the series, were observed to have a severe sensitivity reaction resembling that encountered with the sulfonamides.

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RENAL OXYGEN CONSUMPTION IN MAN DURING ABDOMINAL COMPRESSION

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The renal blood flow, glomerular filtration rate and maximal rates of glucose reabsorption and diodrast excretion are greatly reduced in man by increased intra-abdominal pressure (1). These effects may be attributed to simultaneous elevations in renal venous pressure and intra-pelvic pressure which act respectively to decrease blood flow and to halt urine flow from nephrons which have relatively low terminal intra-luminal pressures. Thus a large proportion of the renal parenchyma apparently no longer functions in producing urine. It seemed of interest to determine whether renal oxygen consumption is altered under these conditions.

METHODS

Eleven convalescent patients, ranging in age from 18 to 36 years, five males and six females, were studied in the resting basal state. In five subjects, renal blood flow and renal oxygen consumption were determined before, during, and following compression of the abdomen by a pneumatic girdle inflated at 80 mm. Hg. In the remainder, only the renal venous oxygen concentrations were obtained under the same conditions.

Determination of renal blood flow. Renal blood flow was estimated as the sodium p-aminohippurate (PAH)² clearance corrected by the value for renal extraction of PAH. The techniques described at length by Goldring and Chasis (2) were employed in the determination of PAH clearances. Urine was collected by catheterization and the bladder washed out with isotonic saline solution during two or more 10- to 15-minute periods before, during, and after compression. Blood samples were collected from a peripheral vein or artery at approximately 30-minute intervals and the values used in the calculation of clearances were determined by interpolation. Aliquots of appropriately diluted urine samples and plasma filtrates, prepared by the method of Fujita and Iwatake (3), were analyzed for PAH by the method of Smith and his co-workers (4).

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² Sodium p-aminohippurate was supplied in ampules of 20 per cent sterile, pyrogen-free solution, through the courtesy of Sharp and Dohme, Inc., Glenolden, Pennsylvania.

Renal venous blood was sampled by the method of venous catheterization for the purpose of determining PAH extraction. An extra-length radio-opaque³ ureteral catheter, washed out constantly with a slow infusion of sterile isotonic saline solution to prevent blockage by clotting, was introduced into a median basilic vein under local anesthesia. It was passed under fluoroscopic control through the superior vena cava and the right atrium into the inferior vena cava. Here the curved tip (Courmand tip) of the catheter was directed to the right and passed into the right renal vein. In the proper position, it could be visualized in the right side of the abdominal cavity, caudad to the shadow of the liver. The right renal vein was always used as a source of renal venous blood because the left spermatic or ovarian vein introduces a relatively large quantity of non-renal venous blood into the left renal vein. Renal extraction of PAH was determined as the difference in PAH concentration in the renal venous and peripheral arterial or venous blood divided by the concentration in peripheral blood.

The concentration of PAH in renal venous blood was measured after a known amount of standard had been added to the plasma filtrate. This procedure improves recovery from plasma of PAH when present in low concentration. No evidence of movement of PAH into or out of erythrocytes was noted even when the plasma and cells remained mixed for several hours after sampling.

Hematocrits of peripheral arterial and renal venous blood were determined in duplicate on blood sampled for oxygen content, using Wintrobe tubes centrifuged at 3000 r.p.m. for one hour. Hematocrit values were corrected by a factor of 0.915 to allow for an error of 8.5 per cent due to the trapping of plasma between cells during centrifugation (5). The average arterial hematocrit was employed in calculating the volume of whole blood flowing through the kidney.

Renal blood flow was calculated from these values as follows:

$$RBF = \frac{UV}{P} \times \frac{1}{1 - Hd} \times \frac{1}{E}$$

where RBF is the renal blood flow in ml. per minute; U, the urinary, and P, the arterial plasma concentration of PAH in mgm. per cent; V, the volume of urine formed in ml. per minute; Hd, the average arterial hematocrit; and E, the renal extraction of PAH, in per cent.

Determination of renal oxygen consumption. Values of renal venous and peripheral arterial oxygen concen-

³ This catheter is manufactured by the United States Catheter and Instrument Company, Glen Falls, New York.

trations were obtained as close as possible to the midpoint of the urine collection period preceding the application of pressure, at the midpoint of the period of compression, and within 30 minutes after the pressure was released. These figures were used in calculating oxygen consumption of the kidney on the basis of averaged renal blood flow during each of the three phases.

The blood samples for oxygen determination were collected anaerobically in oiled, heparinized syringes, and stored under mercury in a refrigerator. The analyses were carried out within several hours. No measurable change in concentration occurred during this period.

Oxygen was determined in 1.0-ml. samples by the method of Van Slyke and Neill (6). Duplicate analyses agreed within 0.1 volume per cent. In order to eliminate variations in observed oxygen content due to changes in hematocrits as a result of dilution during sampling or during passage through the kidney, rather than to changes in oxygen extraction, all values were corrected to a constant hematocrit level, as follows:

corrected oxygen content = $O_2 \times \frac{Hct_A}{Hct}$

where O_2 is the observed oxygen content of the blood sample; Hct its hematocrit; and Hct_A the average arterial hematocrit. This correction was small, usually amounting to less than one per cent of the oxygen content.

The difference between the corrected values of oxygen content of peripheral arterial and renal venous blood in volumes per cent was multiplied by the renal blood flow in hundreds of milliliters per minute to obtain the renal oxygen consumption in milliliters per minute.

In six subjects renal venous oxygen contents alone were measured before, during, and after compression.

These figures have not been corrected by hematocrit values.

RESULTS

The renal blood flow always fell during compression and returned to the control level after release of pressure (Table I). The renal extraction of PAH remained relatively constant. It is possible that the decrements in extraction during "recovery" in J. B. and B. E. resulted from displacement of the catheter by the experimental procedure with contamination of the sample by blood from the inferior vena cava. Since there was greater difficulty keeping the catheter in place in J. B. than in any of the other subjects, the findings in J. B. are particularly suspect.

The renal arteriovenous oxygen difference was not altered in any consistent manner by compression, remaining relatively constant in J. S., B. E., and F. S., falling in M. T. and rising in J. B. (Table I). Further studies of renal venous oxygen content in six other subjects (Table II) likewise failed to reveal any consistent or statistically significant change. Though the technical difficulties mentioned above may have influenced the results in J. B., accounting for the depression in renal venous oxygen content and the elevation in renal arteriovenous oxygen difference, it is

TABLE I
Renal oxygen consumption during abdominal compression

Subject	Renal extraction of PAH	Oxygen content		A-V	Renal blood flow		Renal oxygen consumption		Procedure
		Arterial	Venous						
	<i>per cent</i>	<i>vols. per cent</i>	<i>vols. per cent</i>	<i>vols. per cent</i>	<i>ml. per minute</i>	<i>per cent change</i>	<i>ml. per minute</i>	<i>per cent change</i>	
J. B.	89.8	15.2	14.5	0.6	925	-46	6.0	+69	Control Pressure Recovery
	89.0	15.1	13.1	2.0	502		10.2		
	83.5	14.9	14.2	0.7	790		5.6		
M. T.	92.5	14.5	13.6	0.9	849	-73	7.6	-84	Control Pressure Recovery
	94.6	14.3	13.8	0.5	229		1.2		
	96.0	14.5	13.4	1.0	928		9.7		
J. S.	92.2	18.0	17.1	0.9	1115	-62	10.4	-65	Control Pressure Recovery
	99.0	18.3	17.4	0.8	425		3.6		
	96.4	18.3	17.2	1.1	1156		13.3		
B. E.	95.7	16.1	14.5	1.6	815	-21	13.0	-29	Control Pressure Recovery
	94.8	15.9	14.5	1.4	640		9.3		
	90.8	16.3	14.7	1.5	956		14.8		
F. S.	88.0	15.3	13.9	1.4	1025	-59	14.2	-56	Control Pressure Recovery
	87.5	15.3	13.9	1.5	418		6.2		
	90.7	*	*	*	*		*		

* These values were not obtained because of technical difficulties.

TABLE II

Renal venous oxygen content during abdominal compression

Subject	Renal venous oxygen content ml. per cent		
	Control	Pressure	Recovery
W. F.	14.5	14.5	
E. R.	13.9	14.3	14.4
J. D.	19.5	19.8	19.6
F. M.	17.2	17.1	17.4
L. J.	16.9	17.7	16.9
G. C.	13.9	14.3	14.4

impossible to demonstrate conclusively that the pattern of behavior in J. B. differs significantly from that of the other members of the series. For this reason, elimination of J. B. from consideration was deemed inadmissible.

The consumption of oxygen by the kidneys averaged 10.3 ml. per minute in the control resting state, ranging from 6.0 ml. per minute to 14.2 ml. per minute. With compression it fell to 6.1 ml. per minute on the average, a reduction of 41 per cent. At the same time, the renal blood flow was reduced on the average by 52 per cent. This parallel reduction in renal blood flow and renal oxygen consumption occurred in every subject except J. B. Following release of the pressure all values returned to, or close to, the initial figures.

In association with these changes, the urine flow decreased sharply and the urinary concentration of PAH rose.

DISCUSSION

The renal oxygen consumption is generally believed to be chiefly a function of the blood flow through the kidney (7, 8). Van Slyke and his co-workers (7) found this relationship in normal unoperated dogs over a wide range of blood flow and in common with other workers (9, 10), failed to observe any change referable to alterations in the rate of urine formation. However, the importance of renal arterial pressure changes have been emphasized by Kramer and Winton (11). In a study of oxygen consumption by the explanted canine kidney, these investigators found that alterations in the perfusion pressure produced more or less equivalent changes in oxygen uptake. When blood flow varied spontaneously,

independently of perfusion pressure, renal oxygen consumption was not altered.

There is no reason to believe that the renal arterial pressure is affected by abdominal compression at 80 mm. Hg since intra-abdominal pressure rises no more than 20 mm. Hg, well below the diastolic pressure in the renal artery (1). In addition, it is probable that vasomotor changes are not concerned in the reduction of renal blood flow, although the available facts may be interpreted as evidence of total ischemia in part of the renal vascular bed with "functional amputation" of a portion of renal parenchyma. In this view, the proportionate reduction in oxygen consumption, blood flow, filtration rate and maximal tubular capacities would result from excluding from perfusion a certain fraction of functional renal tissue. However, it has been pointed out elsewhere (1) that this hypothesis requires simultaneous vasodilatation and vasoconstriction in the kidney, and may be set aside as unlikely. Thus it seems permissible to consider oxygen consumption in terms of the kidney as a whole, dismissing the possibility that perfusion pressure may be important in this situation.

The relationship between renal blood flow and oxygen consumption observed by Van Slyke and others in the dog, appears in these data obtained in man. It is possible that this association arises from the diminished delivery of oxygen to the kidney with subsequent reduction of the activity of oxidative enzyme systems in tubular cells. These systems have been found to be extremely sensitive to environmental oxygen tension (12), responding to slight changes by marked reduction in activity. However, the oxygen content of renal venous blood does not change significantly during abdominal compression and it seems unlikely that there is any change in the oxygen tension of renal interstitial fluids. It is interesting that reduced oxygen consumption during abdominal compression is associated with apparent cessation of urine formation in a certain proportion of nephrons (1). The possibility that renal oxygen consumption depends upon the relative proportion of tubular tissue actively functioning in the formation of urine from glomerular filtrate cannot be excluded as an explanation of the data presented here.

SUMMARY

The renal oxygen consumption has been measured directly in normal resting human subjects before, during, and after abdominal compression at 80 mm. Hg.

Resting control oxygen consumption averaged 10.3 ml. per minute in five subjects, ranging from 6.0 ml. per minute to 14.2 ml. per minute.

Abdominal compression reduced renal oxygen consumption to 6.1 ml. per minute on the average, or by 41 per cent. At the same time the renal blood flow was reduced, on the average by 52 per cent. There was no significant change in renal venous oxygen content.

ACKNOWLEDGMENT

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THE EFFECT OF EXERCISE ON RENAL PLASMA FLOW IN NORMAL MALE SUBJECTS

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The effect of exercise on renal function has been the target of numerous research projects, many of which have recently been reviewed by Herlitzka (1). It is generally agreed that exercise exerts an antidiuretic effect, that it depresses urea clearance, and that it increases the specific gravity of the urine. With the introduction of diodrast and of para-amino hippuric acid it became possible to estimate renal plasma flow with considerable accuracy, but these newer methods have not been applied extensively to the question of the effect of exercise.

In order to obtain quantitative information on the phenomenon a method was devised for following changes in renal plasma flow before, during, and after standard exercise of varying degrees of severity. The present report is concerned with the application of this test to normal young men.

METHOD

The subjects were required to omit the meal preceding the test and were not allowed to smoke for at least an hour before the experiment began. They were kept at complete bedrest for 40 minutes or longer before the beginning of the first basal period. Every effort short of the use of sedative drugs was made to allay anxiety.

The technique used is based on that originally described by Chasis *et al.* (2), but embodies certain important modifications made necessary by the test conditions. A constant injection apparatus similar to that described by Earle and Berliner (3) was substituted for the usual gravity type intravenous injection equipment. This device delivers exactly 0.966 cc. test solution per minute with great precision, not only during rest but also during exercise. Samples of urine were collected by voluntary micturition. In order to facilitate the collection of samples of urine, moderate water diuresis (urine flow of 10 to 15 cc. per minute) was instituted and the collection periods were lengthened to 20 minutes.

For the exercise, a motor-driven treadmill was used. No special training period was imposed but those subjects not already familiar with the treadmill were given short trials before experimental observations were begun. Three levels of activity were used: A. 3 m.p.h. at zero per cent grade; B. 3 m.p.h. at 5 per cent grade; and C. 3.5 m.p.h.

at 10 per cent grade. The energy outputs at these levels of work, expressed as oxygen consumption per square meter body surface per minute, are 419, 612 and 1070 cc. The first two levels placed no great strain even on a strictly sedentary individual. The third and highest level taxed normal, active male students only moderately but caused considerable discomfort in subjects accustomed to little or no strenuous exertion. The arm being used for injection of the clearance substance was kept in a relatively fixed position during exercise by having the subject rest his hand on the edge of a shelf placed directly in front of him and adjusted to a point several inches below the level of the shoulders. With the arm thus partly immobilized, the energy outputs for a given treadmill speed and grade are about one to 2 per cent less than if the arms are allowed to swing free. The results, however, are comparable from experiment to experiment since they were obtained under identical conditions; the only variable was the level of exercise being investigated.

The conditions under which renal plasma flow was followed during recovery were the same as those used for determining basal values. The subject walked a few feet to the bed after completing the work on the treadmill, and was required to lie immobile for the rest of the experiment. The constant injection apparatus was moved with the patient without interrupting the injection of the test substance.

The time sequence and complete data for a typical experiment in this series are given in Table I.

MATERIAL

Nine subjects were employed in the study. All were healthy male university students or instructors aged from 21 to 32 years. There was no evidence of renal disease in their histories or in the results of their urinalyses. Resting blood pressures were within the normal range in the entire group.

All clearance values were corrected to 1.73 sq. m. of body surface using the formula of Dubois for total body surface.

Basal values were determined for two or three consecutive clearance periods in each experiment, the usual number being three. More than one experiment was done with eight of the nine subjects, and in six subjects, experiments were repeated six or more times. In order to gain some insight into the reliability of the method, all available basal data were utilized, regardless of variation between consecutive clearance periods or between exp-

TABLE I

Sample experiment (Subject CBC, 24 November 1947; body surface 1.89 sq. m.). P = mgm. PAH in 100 cc. plasma. U = mgm. PAH in 1 cc. urine. V = volume of urine per minute

Time	Procedure	P	U	V	UV	Plasma flow	
						Uncorrected	Corrected*
-30:00	Subject at complete rest	0.10					
-08:00	200 cc. water by mouth						
-01:00	Plasma sample No. 1						
00:00	Begin constant injection (PAH 1.88 gms. per cent)						
02:00	Begin injection priming dose (0.8 gms.)	3.30					
04:00	End injection priming dose						
30:00	Urine sample No. 1 (discarded)						
31:00	Plasma sample No. 2						
32:00	200 cc. water by mouth	3.01	3.97	5.6	22.2	700	641
42:00	200 cc. water by mouth						
50:00	Urine sample No. 2						
51:10	Plasma sample No. 3						
57:00	200 cc. water by mouth	2.97	2.03	9.1	18.5	620	567
70:00	Urine sample No. 3						
71:15	Plasma sample No. 4						
73:00	200 cc. water by mouth						
87:00	200 cc. water by mouth	2.94	1.51	11.5	17.4	592	542
90:00	Urine sample No. 4						
91:15	Plasma sample No. 5						
93:00	Begin exercise: 3 m.p.h. at 5 per cent grade						
109:00	End exercise	3.36	1.38	11.5	15.9	506	463
109:40	Urine sample No. 5						
110:45	Plasma sample No. 6						
112:00	200 cc. water by mouth						
113:00	Begin exercise	3.54	1.90	8.6	16.4	479	439
129:00	End exercise						
130:00	Urine sample No. 6						
131:00	Plasma sample No. 7						
132:00	Subject at complete rest	3.06	3.06	5.7	17.5	528	484
150:00	Urine sample No. 7						
151:00	Plasma sample No. 8						
170:00	Urine sample No. 8						
171:10	Plasma sample No. 9	3.09	2.36	7.2	17.0	555	507

* Corrected to 1.73 sq. m. body surface.

arate experiments. This provided 59 experiments and a total of 140 clearance periods.

In order to evaluate the effect of exercise on basal plasma flow, no experiment was considered valid unless it included two or more basal values which were within 10 per cent of each other. This requirement resulted in the loss of several experiments in which only two basal values were determined. When three consecutive basal values were determined at least two figures were invariably obtained which did not exceed the variation allowed. For the studies on the light work load we obtained 12 valid tests on six subjects; for the intermediate load there were 13 valid tests on eight subjects, and for the heavy stress there were 10 valid tests on five subjects.

RESULTS

The method as described yielded an average basal renal plasma flow of 603.7 ± 114 cc. for consecutive duplicate determinations and 613 ± 107

cc. for triplicates. Statistical treatment of the basal data (4) disclosed that repeatability for individual subjects was not high. The error (random variance) represents 57.1 per cent and 48.9 per cent of the interindividual variance in the case of duplicate and triplicate control data, respectively, the corresponding coefficients of consistency (rc) being 0.429 and 0.511. The intraindividual standard deviations ($\sqrt{V_{WI}}$) are ± 89 and ± 78 . For comparison, the corresponding interindividual standard deviations ($\sqrt{V_{WD}}$) are ± 175 and ± 107 , respectively. A trend in both the duplicate and triplicate data was apparent. The successive means for the duplicate determinations were 621.5 cc. and 585.9 cc. and those for the triplicate data were 646.0 cc., 606.0 cc., and 587.7 cc. The differences between these means ap-

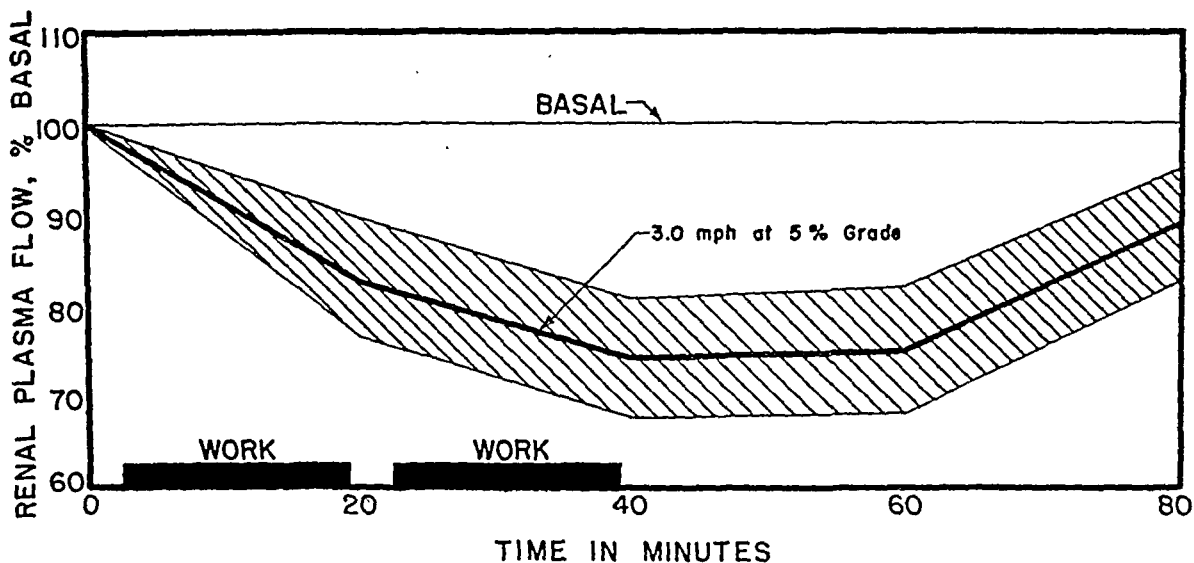


FIG. 1. MEAN CHANGES (HEAVY LINE) IN RENAL PLASMA FLOW DURING AND AFTER THE INTERMEDIATE GRADE OF WORK

The shaded area represents standard deviation.

proach significance for triplicate data and are significant for duplicate data.

The effects of exercise on renal plasma flow as revealed by our technique are set out in Figures 1 and 2, and in Tables I and II. Figures 1 and 2

show that there was a progressive decline in plasma flow as work proceeded. In no instance did the pattern of progressive decline in plasma flow fail to occur. The direct relationship between the amount of decline and the severity of

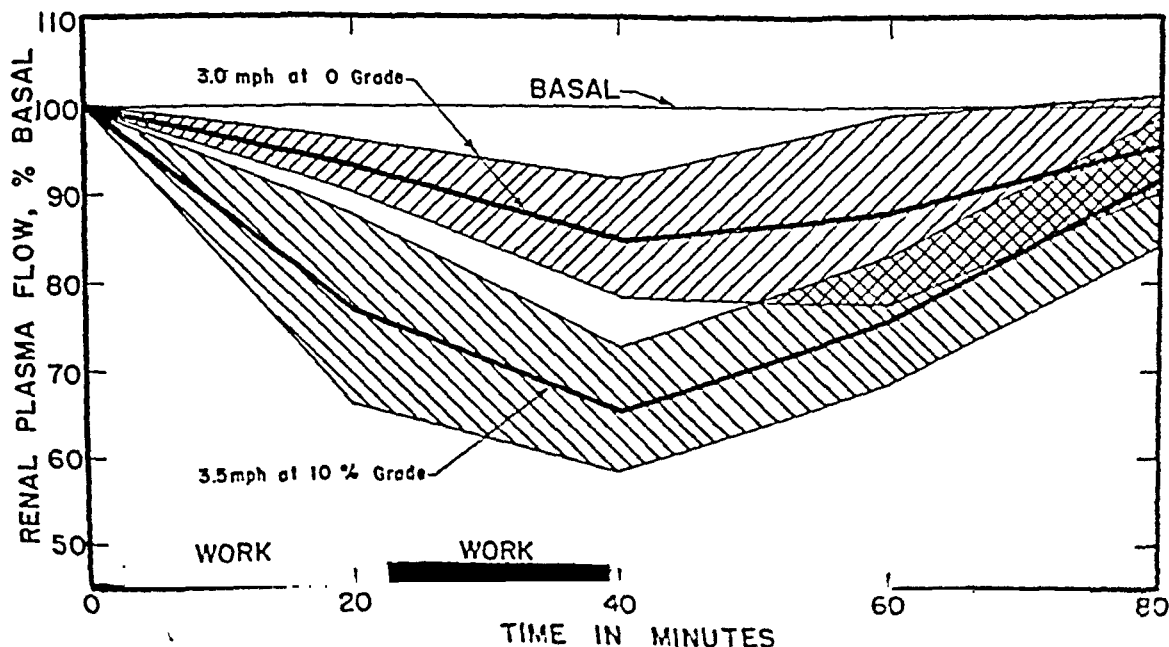


FIG. 2. MEAN CHANGES (HEAVY LINE) IN RENAL PLASMA FLOW DURING AND AFTER THE LIGHTEST AND HEAVIEST GRADES OF WORK

The shaded areas represent standard deviations

TABLE II

Mean renal plasma flow in work and in recovery from work at three levels of activity
All values in cc. per minute for 1.73 sq. m. of body surface

	Basal	Work 1	Work 2	Rest 1	Rest 2
Stress A	593.3±72.2	551.3±74.5	502.7±64.4	528.8±84.3	572.4±76.9
Stress B	595.7±65.2	491.7±48.3	438.1±27.1	442.2±52.7	516.2±42.3
Stress C	576.4±68.1	435.4±42.8	375.5±49.5	459.7±72.1	540.8±60.1

exercise is clearly apparent, although there is considerable overlapping when the standard deviations are plotted along with the means.

Recovery of the decline in renal plasma flow to exercise is less predictable. With the light-work load recovery was still incomplete 40 minutes after cessation of work, the residual decrement at that point showing a statistically significant difference from the basal value. The same was true for the heavier loads, but the amount of residual decrement bore no relation to the severity of the work.

In evaluating statistically the significance of changes under experimental conditions an F-test for paired variates was employed. Both the magnitude of the changes and individual variation in response were taken into account. For each level of work, the differences between the basal renal plasma flow on one hand and those during work and recovery from work on the other proved to be significant or highly significant (< 5 per cent, < 1 per cent, respectively). In evaluating the effect of increasing the severity of the work the differences observed at the lightest and the heaviest work loads (3 m.p.h. at zero grade, and 3.5 m.p.h. at 10 per cent grade, respectively) were tested by means of the same F-test. The differences were significant or highly significant throughout.

DISCUSSION

The two values for mean basal renal plasma flow in normal males as determined in this study (593.3 ± 72.2 cc. for duplicate and 613 ± 107 cc. for triplicate determination) are comparable to those reported by Merrill (5) who obtained a value of 626 ± 165 cc. in 39 experiments on 35 normal individuals (males and females), and to that obtained by Heller and Jacobson (6) which was 584.4 ± 84.4 cc. in eight normal males. Para-nitrohippuric acid was used as the clearance sub-

stance in all three studies but in the latter two, urethral catheterization was used for collection of samples of urine. Although the collection of urine samples by voluntary micturition instead of catheterization does not appear to have introduced serious error, it obviously limits the use of the method to subjects who have reasonable control over the initiation of micturition. It also imposes the necessity for working at relatively high rates of urine flow.

The possibility that water diuresis, itself a stress of a sort, may exert an independent action on renal plasma flow has never been adequately studied in the human subject, but Dicker and Heller (7) found that inulin and diodone clearances remained reasonably constant in rats at widely varying rates of urine flow. They state that "in the dog, as in man, diodone and inulin clearances do not rise with increase in urine flow." In our work no evidence of a trend was found when the corrected basal clearance values were plotted against the rates of urine flow. The mean rate of urine flow during basal determinations was 13.0 ± 4.4 cc. per minute.

The slight trend in consecutive basal values finds no ready explanation and is currently under further experimental investigation. It does not appear to be related to rates of urine flow. The same phenomenon is apparent in Benzinger's data (8) for three consecutive urea clearance values under basal conditions. In our own work there was a definite tendency for the clearance values to level off in the second and third basal periods and the basal value used for comparison with the values obtained during application of the stress was a mean, usually of the second and third basal figures. The use of such a mean value largely, if not entirely, compensated for the effect of the trend.

Barclay and co-workers (9) measured inulin and diodrast clearances in eight normal subjects immediately after cessation of exercise (440 yard dash) and found that the renal plasma flow fell from 18 to 54 per cent of the resting values; ten to 40 minutes were required for the renal plasma flow to return to resting levels. The effect of sustained exercise was studied by means of a bicycle ergometer in two subjects and the renal plasma flow was found to decline steadily during the work, the duration and severity of which were not specified. White and Rolf (10) noted little change in renal plasma flow during light exercise but found it to fall markedly during heavy exercise. Merrill and Cargill (11) obtained a similar result in normal individuals performing work in both the upright and recumbent positions. Our results are generally in accord with those obtained by these workers but demonstrate more clearly that the extent of the decline in renal plasma flow is directly related to the severity of the exercise within certain, as yet incompletely defined, limits.

Recovery of renal plasma flow from the effects of exercise is considerably slower than is recovery as judged by pulse rate and blood pressure. It appears from our results that the rate and extent of recovery are not directly related to the severity of the work and that plasma flow is usually still somewhat depressed even after 40 minutes of rest following all three grades of exercise.

The mechanism by means of which exercise depresses renal plasma flow is uncertain and our work throws no new light on it. Barclay *et al.* (12) found that in exercise there is depression of the glomerular filtration rate and filtration fraction as well as of plasma flow. Since adrenalin, like pain and apprehension, produces an increase in filtration fraction while depressing plasma flow, they reason that the exercise effect is not mediated by adrenalin.

The chief significance of the diversion of blood from the kidney during exercise may be that more blood is made available for working muscles, a view suggested by Edwards *et al.* (13), among others. From our experience with two consecutive periods of exercise at the lightest work load, about 150 cc. of whole blood per minute are diverted from the kidney for circulation elsewhere. For the heaviest work load, the corresponding

figure is about 330 cc., a not inconsiderable amount.

SUMMARY

1. Renal plasma flow was estimated from para-aminohippurate clearance in nine healthy men aged 21 to 32 years. In 59 experiments, providing 140 clearance periods, the basal mean for consecutive determinations in triplicate was 613 ± 107 cc. per minute, corrected to 1.73 sq. m. body surface. Variability was analysed into interindividual, intraindividual, and random components.

2. During the first 16 minutes of walking on the motor-driven treadmill the renal plasma flow was decreased by an average of 6, 17 and 25 per cent (of the resting control) at work levels, respectively, of 3.0 m.p.h. at zero grade, 3 m.p.h. at 5 per cent grade, and 3.5 m.p.h. at 10 per cent grade.

3. Continued walking for another 16 minutes yielded corresponding average reductions of 15, 27 and 35 per cent of the resting control values.

4. Return of the renal plasma flow to basal levels was incomplete after 40 minutes of recovery following this exercise.

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THE RENAL CLEARANCE OF ENDOGENOUS "CREATININE" IN MAN

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The existing methods for the analysis of inulin, as well as the requirements for the continuous intravenous infusion of this material, place the inulin clearance method for measuring the glomerular filtration rate beyond the reach of practicality for many laboratories and in general limit clearance observations to relatively short intervals of time. There is a requirement for a simpler clearance method of at least approximate accuracy by which observations can be made continuously for periods of 24 hours or longer. Endogenous "creatinine" appears to offer possibilities in this direction, since it would obviate the necessity for intravenous infusions, frequent withdrawal of blood and elaborate analytical procedures.

The nature of the endogenous substance or substances which yield color with Jaffe's alkaline picrate reaction for creatinine has long been a matter of controversy. The literature on this subject is reviewed by Miller and Dubos (1, 2). These authors, using an allegedly specific enzymatic method on 1:5 plasma filtrates prepared with 10 per cent sodium tungstate and 0.66 N sulfuric acid, concluded that in normal human plasma, creatinine constitutes 80 to 100 per cent of the chromogenic substance, although in renal disease, where the chromogen is elevated, the plasma may contain relatively large amounts of non-creatinine chromogenic material.

Exogenous creatinine is excreted in man both by glomerular filtration and tubular secretion. Crawford (3) has recently adduced new evidence on this point by showing that the exogenous creatinine/inulin and the exogenous creatinine/thio-sulfate clearance ratios are reduced by saturation

of the tubules with diodrast or p-aminohippuric acid. Although Crawford's control ratios are not as high as those reported by others, the average value as recorded by her and by Shannon (4), Shannon and Ranges (5), McCance and Widdowson (6 to 8), Josephson and Godin (9), and Miller and Winkler (10) appears to be about 1.3, indicating that about 30 per cent of the urinary exogenous creatinine is excreted by the tubules.

Several investigators have reported, however, that the endogenous chromogen/inulin clearance ratio in subjects without renal disease is close to 1.0. Miller and Winkler (10), using the enzymatic method of Miller and Dubos (2), report ratios varying from 0.8 to 1.5 (average 1.07) and exogenous/endogenous chromogen ratios, as determined in successive clearance periods, ranging from 1.1 to 2.0 (average 1.63).

Popper and Mandel (11) report the endogenous chromogen/xylose clearance ratio to be 1.35, a value comparing favorably with the average inulin/xylose clearance ratio of 1.25 (12), but Popper and Mandel's ratios vary from 0.77 to 4.5, indicating great uncertainty either in the clearance ratio or in their analytical methods. The method used by Popper and Mandel was that of Popper, Mandel and Mayer (13), in which the plasma proteins were precipitated with picric acid.³

Steinitz and Turkänd (15), using Popper, Mandel and Mayer's picric acid filtrate, report a chromogen/inulin clearance ratio for normal subjects ranging from 0.73 to 1.17 (average 1.03). (The exogenous/endogenous chromogen ratio as deter-

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³ Findley (14) found that the exogenous creatinine clearance in man is independent of plasma concentration only when an endogenous blank of about 0.5 mgm. per cent is deducted from the total chromogen value, but the order of magnitude of the correction seems too small to warrant this interpretation. A critical answer to this question can only be obtained by the study of simultaneous clearances.

mined before and after the administration of creatinine in subjects with and without renal disease ranged from 0.92 to 1.87 and averaged 1.22.) Since the normal values obtained with picric acid filtrate lay between 0.5 and 1.0 mgm. per cent, while those reported with the Folin tungstate filtrate were 1 to 2 mgm. per cent, Steinitz and Turkänd suggested that the picric acid used in precipitation of the proteins precipitates some of the non-creatinine chromogenic substance. However, the figures of 1 to 2 mgm. per cent as originally reported by Folin and Denis (16) were obtained with whole blood. The red cells are now known to contain considerable quantities of non-creatinine chromogen. With plasma, the tungstic acid filtrate in our hands yields values varying from 0.64 to 1.10 mgm. per cent in subjects without renal disease.

Smith, Finkelstein and Smith (17), using the picric acid filtrate, found an endogenous chromogen/inulin clearance ratio ranging from 1.01 to 1.42 (average 1.19), whereas using the Steiner, Urban and West filtrate ($\text{BaCO}_3\text{-Fe}_2[\text{SO}_4]_3$) this ratio ranged from 0.81 to 1.08 and averaged 0.94. In all cases these ratios were depressed slightly, though scarcely beyond the limits of analytical error, during saturation of the tubules with diodrast. Although both methods of protein precipitation gave quantitative recovery of added creatinine, the endogenous chromogen in the picric acid filtrate from a series of 14 samples of human plasma ranged from 50 to 79.2 (average 66.6) per cent of that present in the iron filtrate. (This discrepancy might in part be due to the greater acidity of the picric acid filtrate.)

In four subjects with renal disease, Miller and Winkler (10) report endogenous chromogen/inulin clearance ratios of 0.9 to 1.7 (average 1.38) and an exogenous/endogenous chromogen clearance ratio in successive clearance determinations of 1.0 to 1.7 (average 1.3). Similarly Steinitz and Turkänd (15) found that in subjects with glomerulonephritis the endogenous chromogen/inulin clearance ratio ranged from 1.04 to 1.73 (average 1.37).

It appears from the above data that in subjects with reduced filtration rates owing to renal disease the endogenous chromogen clearance substantially exceeds the inulin clearance and therefore affords no reliable index of the filtration rate. However,

the average figures on subjects without renal disease indicate that the endogenous chromogen clearance is close to the filtration rate, despite the fact that the variability in the clearance ratio indicates variation in the composition of the chromogen and, as the determinations have hitherto been carried out, the data leave a serious question of reliability in any one subject. Since the methods of determining the endogenous chromogen have differed considerably, it seemed to us that these variations might in part be attributable to differences in technique, and we have therefore reexamined the endogenous chromogen clearance, comparing it with the inulin clearance and the thiosulfate clearance (which Newman, Gilman and Philips [18] have shown to be equal to inulin clearance in man) and in some instances with the mannitol⁴ and exogenous creatinine clearances.

METHODS

Standard renal clearance procedures (22) were utilized. One liter of tap water was administered one to two hours before the beginning of the test. Urine was collected by an in-dwelling catheter and the bladder was rinsed with saline and emptied with air at the end of each urine collection period. Priming and maintenance dosages of test substances were calculated to yield plasma concentrations of 15 to 20 mgm. per cent of inulin, 25 to 30 mgm. per cent of thiosulfate, 200 to 300 mgm. per cent of mannitol, 25 mgm. per cent of exogenous creatinine, 2 to 3 mgm. per cent of PAH for effective renal plasma flow and 60 mgm. per cent for Tm_{PAH} .

A control urine (U_0) was collected in each instance to permit the determination of the excretion of blank substances (U_0V), for which correction was made in the calculation of clearances. This blank correction is particularly important where there is reduced renal function. In one of our patients with marked nitrogen retention in whom the filtration rate ranged from 2 to 4 cc. per minute the inulinoid blank was 26 per cent and the thiosulfate blank 33 per cent of the quantities excreted during clearance determinations.

The number of urine collection periods varied from three to nine and were usually about ten minutes in dura-

⁴ Several investigators (Berger, Farber and Earle [19]; Corcoran and Page [20]; Hoobler [21]) have reported that the mannitol clearance as determined with the periodate-chromotropic acid method of Corcoran and Page (20) is some 10 per cent less than the inulin clearance, an observation which has been confirmed in this laboratory, but we include our mannitol data here since they still afford useful evidence on the endogenous chromogen clearance. For the calculation of clearance ratios the mannitol figures are multiplied by 1.10 to approximate the hypothetical inulin clearance.

tion, except when low urine flow required extension to 15 to 20 minutes. Two blood specimens were drawn for each three urine collection periods. Blood concentrations were plotted semilogarithmically against time and mean concentrations estimated by interpolation to a point 2.5 minutes⁵ preceding the midpoint of each collection period.

In six subjects clearances of endogenous chromogen and inulin were studied over a period of 24 to 48 hours. The urine collection periods were four hours in length and urine samples were obtained by voiding. Blood samples were drawn at the midpoint of each urine collection period. The number of periods varied from five to 12.⁶

Inulin was determined by a modification of Harrison's method as described by Goldring and Chasis (23), with the exception that 2 cc. of undiluted plasma were added to 6 cc. of 20 per cent yeast, 1.0 N NaOH was substituted for 1.1 NaOH in precipitation, and B_0 was determined by adding a known amount of inulin to the B_0 filtrate. In ten experiments inulin recoveries were performed simultaneously with the analyses and these ranged from 95 to 100.5 per cent with an average of 98.5 per cent.

In initial experiments we obtained only 90 per cent recovery from plasma of added creatinine when using the picric acid precipitation method of Popper, Mandel and Mayer (13). Consequently we turned to different protein precipitation methods. We found a modified Folin-Wu tungstic acid method, using a 1:4 dilution, best suited to our purposes. Six cc. of plasma were diluted with 6 cc. of water and the proteins precipitated by the addition of 6 cc. of 5 per cent $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ and 6 cc. of 0.66 N H_2SO_4 added dropwise, the mixture being shaken intermittently and allowed to stand for ten minutes, after which time it was filtered through washed cotton. The recovery of added creatinine was checked by substituting 6 cc. of creatinine standard solutions, ranging from 1.0 to 2.5 mgm. per cent, for the 6 cc. of water. A reference standard was prepared by adding 6 cc. of creatinine standard to 18 cc. of water. Six-cc. samples of the filtrates and creatinine standard (in duplicate) were subjected to the Jaffé reaction according to the modification advocated by Bonsnes and Taussky (24). In 25 such determinations, where the endogenous chromogen ranged from 0.72 to 2.48 mgm. per cent and the added creatinine from 1 to 2.64 mgm. per cent, the recovery averaged 100.2 per cent, with one value of 91.6 and all others lying between 98.5 and 106 per cent.

The quantity of acid used in the above method is twice that usually recommended for the tungstic acid filtrate. The use of 0.33 N H_2SO_4 instead of 0.66 N H_2SO_4 in the 1:4 dilution precipitation results in endogenous chro-

mogen values which agree closely with those found in the more acid filtrate, but the recoveries of added creatinine are significantly lower. In a series of six such recoveries the average was close to 90 per cent with extremes of 83.0 and 93.5 per cent.

Thiosulfate was determined by the method of Newman, Gilman and Philips (18). PAH was determined by the method of Smith *et al.* (25) and mannitol by the method of Corcoran and Page (20). In ten trials the plasma recovery of mannitol using this method ranged from 96.7 to 103.5 per cent, and averaged 99.1 per cent.

The Coleman Jr. spectrophotometer was used for PAH and creatinine determinations and the Evelyn photoelectric colorimeter for mannitol and inulin.

All subjects were patients from the wards of the Third (New York University) Medical or Surgical Services of Bellevue Hospital.

RESULTS

Endogenous "creatinine" chromogen clearances in individuals without renal disease. The results of observations on 14 subjects without renal disease are summarized in Table I. The individuals studied are in the younger adult and middle-aged groups. The plasma concentrations of the test substances were all in the proper range and were usually declining slightly throughout the period of observation, except for endogenous chromogen which remained practically constant in any one individual. The endogenous chromogen plasma concentration ranged from 0.64 to 1.10 mgm. per cent, with an average of 0.91 mgm. per cent.

The endogenous chromogen/inulin clearance ratios in these subjects ranged from 0.88 to 1.10 with an average in 94 clearance periods of 1.00 ± 0.018 . Except for subjects No. 7 and No. 11 agreement between the two clearances was uniformly good, not only in the average but also in single urine collection periods. In the two cases mentioned the endogenous chromogen clearances averaged 10 per cent higher and 12 per cent lower, respectively, than the inulin clearances. With subject No. 7, the 10 per cent disparity may in part be attributable to analytical errors in the inulin clearance, since the simultaneous thiosulfate clearances averaged only 4 per cent lower than the endogenous chromogen clearances. Simultaneous thiosulfate clearances were not performed in subject No. 11. The endogenous chromogen/thiosulfate clearance ratios in seven subjects ranged from 0.84 to 1.01 and averaged 0.95 ± 0.018 representing a total of 47 clearance periods.

⁵ In view of T. Hilden's recent studies (personal communication) it appears that this figure should be six minutes for a urine flow of 2 to 10 cc. per minute.

⁶ We are indebted to Dr. David Baldwin and Dr. Herman Villareal for their assistance with these latter experiments. The details of technique as well as the 24-hour cyclic variation of renal function will be reported elsewhere.

TABLE I

Comparison of simultaneous endogenous "creatinine" chromogen, inulin and thiosulfate clearances in patients with normal renal function

No.	Pa-tient	Sex	Age	Diagnosis	No. of pe-riods	Mean plasma concentration			Plasma clearance*			Clearance ratios			
						Inulin	"Creat."	Thio.	Inulin	"Creat."	Thio.	"Creatinine" inulin		"Creatinine" thio.	
												Average	Range	Average	Range
1	H. K.	M	57	Psychoneurosis	3	mgm./100 cc.	mgm./100 cc.	mgm./100 cc.	cc./min.	cc./min.	cc./min.	0.95	0.90		
2	S. J.	F	23	Alcoholic gastritis	4	23.0	0.96	29.0	95.6	99.5	103	1.00	0.98	1.01	0.93
3	C. L.	M	42	Convalescent pneumonia	7	17.0	0.80	19.5	147.0	141.0	147	1.00	0.98	0.96	1.02
4	J. O.	M	52	Convalescent pneumonia	7	22.5	0.66	22.5	133.0	135.0	138	1.02	1.01	0.97	0.99
5	J. H.	M	20	Psychopathic personality	9	24.1	1.10	24.4	112.0	120.0	128	1.08	0.96	0.96	1.06
6	J. H.	M	37	Normal	7	19.8	0.88	17.5	111.0	114.0	131	1.02	1.05	0.90	0.99
7	J. H.	M	31	(petit mal) Epilepsy	6	27.3	1.04	25.5	94.7	104.0	109	1.10	1.02	0.90	0.88
8	T. R.	M	44	Convalescent pneumonia	6	22.0	0.70	18.9	121.0	115.0	135	0.94	1.14	0.84	0.93
9	F. N.	M	35	Normal	6	18.5	0.88		125.0	120.0		0.96	1.06		0.99
10	G. W.	M	24	Postoperative skin graft	5	9.0	1.00		147.0	154.0		1.05	1.09		0.91
11	J. C.	M	20	Lymphopathia venereum	8	11.2	0.92		138.0	119.0		0.88	1.15		
12	P. R.	M	28	Healing pelvic fracture	6	11.5	0.64		158.0	152.0		1.01	0.81		
13	D. W.	M	22	Hyperhydrosis	8	13.5	1.00		114.0	113.0		0.99	0.92		
14	G. F.	M	33	Alcoholic	12	15.0	1.08		123.0	121.0		0.99	1.06		
				Total	94					Average		1.00 ±.018	1.10	0.95 ±.018	

* All clearance figures are corrected for a body surface area of 1.73 sq. meters.

Endogenous "creatinine" chromogen clearances in individuals with renal disease. Data from 13 subjects with renal disease are presented in Table II. Three had chronic glomerulonephritis; two, acute glomerulonephritis; three, essential hypertension; two, chronic pyelonephritis; two, diabetes associated with albuminuria; and one, disseminated lupus erythematosus with hematuria. The endogenous chromogen levels ranged from 0.80 to 15.1 mgm. per cent, and the inulin plasma clearances varied from 97 down to 3 cc. per minute.

Exclusive of subject No. 12 the endogenous chromogen/inulin clearance ratios ranged from 0.89 to 1.25 with an average in 57 periods of 1.04 ± 0.109 . In this group a discrepancy between the two clearance values of 10 per cent or greater generally appears in subjects with filtra-

tion rates below 40 cc. per minute. However, in such subjects the absolute differences in the clearance values are of such a small magnitude as to leave the endogenous chromogen clearance a clinically useful test approximating the filtration rate. A single exception to this statement is represented by subject No. 12, a young woman in the nephrotic stage of chronic glomerulonephritis; her inulin, thiosulfate and endogenous chromogen clearances in six periods were 38.8, 39.2 and 62.6 cc./min., respectively, with an endogenous chromogen/inulin clearance ratio of 1.61. This last value was checked after five weeks and the clearance ratio was 1.65.

The thiosulfate clearances revealed wider fluctuations from the endogenous chromogen clearances both in the average and in individual pe-

TABLE II

Comparison of simultaneous endogenous "creatinine" chromogen, inulin and thiosulfate clearances in patients with renal disease

No.	Patient	Sex	Age	Diagnosis	No. of periods	N.P.N.	Mean plasma concentration			Plasma clearance			Clearance ratios			
							Inulin	"Creat."	Thio.	Inulin	"Creat."	Thio.	"Creatinine" inulin		"Creatinine" thio.	
													Average	Range	Average	Range
						mgm./100 cc.	mgm./100 cc.	mgm./100 cc.	mgm./100 cc.	cc./min.	cc./min.	cc./min.				
1	M. G.	F	70	Chronic glomerulonephritis	5	73	35.7	2.46	32.3	21.5	26.8	22.5	1.25	1.19	1.19	1.02
2	A. T.	M	54	Chronic glomerulonephritis; uremia	4	120	17.0	5.21	11.5	8.58	9.55	9.52	1.11	1.35	1.08	1.27
3	H. L.	F	78	Diabetes mellitus; albuminuria	3	30	18.0	0.80		59.9	64.3		1.07	1.04		1.07
4	G. G.	M	18	Acute glomerulonephritis	6	90	10.2	1.12		51.8	53.5		1.03	1.21		1.11
5	K. Z.	F	23	Lupus erythematosus disseminatus	6	30	15.0	0.96	9.0	88.3	89.3	90.0	1.00	1.06	0.99	0.98
6	S. W.	F	22	Acute glomerulonephritis	7	150	15.5	15.1	15.8	3.9	4.53	2.78	1.16	1.08	1.63	1.01
7	J. B.	F	38	Malignant hypertension; uremia	6	121	15.6	9.80	9.2	2.64	3.03	2.07	1.15	1.05	1.46	1.47
8	P. K.	M	45	Essential hypertension; albuminuria	5	35	20.0	1.76	17.0	56.5	54.6	62.2	0.97	1.35	0.88	1.97
9	J. M.	M	47	Hypertension; heart failure; albuminuria	3		25.7	2.80	21.0	44.8	41.0	50.3	0.92	1.09	0.81	0.98
10	E. S.	F	59	Diabetes mellitus; albuminuria	6	32	20.3	1.16	15.1	96.9	86.5	113.0	0.89	0.88	0.77	0.80
11	V. K.	F	38	Chronic pyelonephritis	3		11.6	1.76	16.5	36.6	38.9	34.3	1.06	0.94	1.13	0.85
12	V. S.	F	18	Chronic glomerulonephritis; nephrotic stage	6	16	17.2	1.20	22.6	38.8	62.6	39.2	(1.61)*	1.05	(1.60)*	1.05
13	L. O.	F	39	Chronic pyelonephritis; uremia	3		21.9	9.90		3.78	3.54		0.94	1.07		1.21
				Total	63								1.04 ± .109	1.43	1.10 ± .292	1.65
											Average					

* Omitted from averages.

TABLE III

Endogenous "creatinine" chromogen mannitol clearance ratios compared with successive exogenous creatinine mannitol clearance ratios *

Patient	No.	Sex	Age	Diagnosis	Plasma clearance				Clearance ratios†	
					Endog. creat.	Mannitol	Exog. creat.	Mannitol	Endog. creat. Mannitol	Exog. creat. Mannitol
					cc./min.	cc./min.	cc./min.	cc./min.		
M. M.	1	F	44	Osteoarthritis	91.1	83.3	113.0	78.1	0.99	1.32
M. W.	2	F	30	Bronchitis	102.0	104.0	207.0	115.0	0.89	1.64
A. D.	3	F	60	Neuralgia	85.1	85.2	119.0	78.7	0.91	1.37
R. B.	4	F	22	Bronchial asthma	101.0	120.0	168.0	116.0	0.77	1.32
V. K.†	5	F	38	Chronic pyelonephritis	38.9	36.6	43.5	35.8	1.05	1.21
K. Z.†	6	F	23	Disseminated lupus erythematosus	89.3	88.3	92.2	74.4	1.01	1.24
								Average	0.94	1.35

* All clearance figures represent the averages of at least three clearance periods.

† Inulin instead of mannitol was used in these cases. The figures in the mannitol columns therefore represent inulin clearances.

‡ These ratios are expressed as $\frac{\text{creatinine}}{1.10 \times \text{mannitol}}$ in order to make the figures comparable to the $\frac{\text{creatinine}}{\text{inulin}}$ ratios of patients V. K. and K. Z.

TABLE IV
The effect of caronamide and PAH tubular blockade on the excretion of endogenous "creatinine" chromogen *

Caronamide										PAH tubular blockade									
No.	Patient	Age	Diagnosis	Before caronamide			During caronamide			No.	Patient	Age	Diagnosis	Before Tm _{PAH}			During Tm _{PAH}		
				Clearance		"Creat." Mannitol	Clearance		"Creat." Mannitol					Clearance		"Creat." Inulin	Clearance		"Creat." Inulin
				"Creat." Mann.	cc./min.	cc./min.	cc./min.	cc./min.	cc./min.					cc./min.	cc./min.	cc./min.	cc./min.	cc./min.	
1	M. M.	44	Osteo-arthritis	91.1	83.3	1.10	83.3	86.6	0.96	7	E. S.	59	Diabetes mellitus	83.4	91.2	89.5	103.0	0.87	
2	M. W.	30	Bronchitis	102.0	104.0	0.98	90.1	96.3	0.94	8	P. K.	45	Essential hypertension	57.9	62.0	51.3	50.9	1.01	
3	A. D.	60	Neuralgia	85.1	85.2	1.00	85.1	83.0	1.02	9	J. B.	38	Malignant hypertension; uremia	2.68	2.47	3.38	2.81	1.20	
4	R. B.	22	Bronchial asthma	101.0	120.0	0.84	129.0	135.0	0.95	10	S. W.	22	Acute nephritis	3.79	3.12	5.27	4.69	1.12	
5	V. K.†	68	Chronic pyelonephritis	38.9	36.6	1.05	33.6	37.0	0.91	11	G. G.	18	Acute nephritis	54.0	53.5	53.1	49.1	1.08	
12	(V. S.)††	18	Nephrotic stage; chronic nephritis	62.6	38.1	1.64	41.7	39.0	1.07	12	(V. S.)††	18	Nephrotic stage; chronic nephritis	57.7	36.1	56.7	39.1	1.45	
Average				0.99						Average			1.03			1.06			

* All clearance figures represent the average of at least three clearance periods.

† The figures in the mannitol columns represent inulin clearances in these cases.

‡ Omitted from averages. This case, V. S., is the same patient as No. 12, Table II, in which a marked discrepancy was found between the endogenous creatinine chromogen and inulin clearances.

riods, than did the inulin clearances. The average endogenous chromogen/thiosulfate ratio in 51 periods was 1.10 ± 0.292 with extremes ranging from 0.77 to 1.63. We believe this wide fluctuation is in part attributable to technical and analytical errors related to the use of relatively low thiosulfate blood levels in the presence of nitrogen retention, where the blood and urine thiosulfate blanks, as measured by the Newman, Gilman and Philips method (18), may be markedly elevated.

Exogenous creatinine clearances. The results of simultaneous clearances of exogenous creatinine and mannitol in four normal subjects, and of exogenous creatinine and inulin in two subjects with renal disease, are presented in Table III. These values are compared with endogenous chromogen and mannitol clearances determined just prior to the administration of exogenous creatinine. Our observations confirm those of Shannon (4), Miller and Winkler (10), McCance and Widdowson (6 to 8), Crawford (3), and others; namely, that the exogenous creatinine clearance is considerably in excess of the inulin clearance. Thus, the exogenous creatinine/ $1.10 \times$ mannitol clearance ratio averaged 1.44 as compared to a ratio of 0.94 for endogenous chromogen/mannitol.

The effect of caronamide and tubular blockade by sodium p-aminohippurate on endogenous chromogen clearances. Caronamide⁷ (4-carboxyphenylmethanesulfonanilide) has been shown by Beyer and his coworkers (26) to block the tubular ex-

⁷ Caronamide (Staticin) was kindly supplied by Sharp and Dohme.

cretion of penicillin, phenol red, diodrast and PAH, and the recent studies of Earle and Brodie (27) show that this may be attributable to the fact that caronamide is itself excreted by the tubules. The data in Table IV indicate that, exclusive of subject No. 12, no significant depression of the endogenous chromogen clearances followed the administration of caronamide in doses of 2 grams every four hours for a total of eight doses prior to observation. The average change of the endogenous chromogen/mannitol clearance ratios following caronamide was -3 per cent.

In contrast to the finding of Crawford (3) that the exogenous creatinine/inulin clearance ratio is consistently reduced by saturation of the tubules with p-aminohippurate, the endogenous chromogen/inulin ratio is, in our experience, not significantly changed during T_{mPAH} measurement as compared with control periods (second half of Table IV). The average change of this ratio during T_{mPAH} , exclusive of subject No. 12, was +3 per cent. The failure of caronamide and p-aminohippurate to depress this ratio indicates that there is no significant tubular excretion of the endogenous chromogen in the subjects tested.

In subject No. 12, on the other hand, who consistently showed control endogenous chromogen/inulin clearance ratios of about 1.61, the ratio was depressed to 1.07 following administration of caronamide, and to 1.45 during T_{mPAH} . This depression indicates that in this patient the endogenous chromogen was excreted in a manner similar to exogenous creatinine, namely, by tubular excretion as well as glomerular filtration.

TABLE V
Comparison of simultaneous endogenous creatinine chromogen and mannitol clearances in infants

No.	Patient	Age	Diagnosis	Number of periods	Creatinine plasma concentration	Plasma clearance		Clearance ratio*
						Creatinine	Mannitol	Creatinine/mannitol
1	M. B.	2 mos.	Normal	6	0.56	26.5	49.6	0.59
2	C. S.	24	Mongolian idiot	6	0.68	14.9	24.6	0.55
3	R. W.	2	Normal	6	0.60	10.5	16.4	0.55
4	J. H.	25	Normal	6	0.44	28.7	37.8	0.69
							Average	0.60

*These figures are expressed as $\frac{\text{creatinine}}{1.10 \times \text{mannitol}}$ since cited evidence points to $1.10 \times$ mannitol clearance as being approximately equivalent to the inulin clearance.

Endogenous chromogen clearances in infants. The results obtained with four infants are tabulated in Table V.⁸ The endogenous chromogen/1.10 \times mannitol clearance ratios ranged from 0.55 to 0.69, with an average of 0.60. The plasma levels of endogenous chromogen were low, ranging from 0.44 to 0.68 mgm. per cent, with an average of 0.57 mgm. per cent. It appears that the chromogen has a different composition, or is excreted by the kidney in a different manner, in infants than in adults.

DISCUSSION

Our data indicate that the endogenous "creatinine" chromogen clearance may be used as an index of the filtration rate in normal adults, and as a useful clinical approximation in subjects with renal disease. We recognize that the chromogen is certainly not entirely creatinine, since it is excreted in a different manner than is exogenous creatinine, and that it probably represents more than one substance, its composition possibly varying from time to time. In view of this fact, and in view of the discrepancy between the chromogen clearance and the inulin clearance in subjects with renal disease, we do not advocate the use of the chromogen clearance for precise studies, even in normal subjects; but we do believe that many renal clearance problems, especially protracted studies, can be expedited by its use. It is, moreover, a useful clinical tool for the general evaluation of renal disease. The time required for the analyses of specimens obtained from six clearance periods using endogenous "creatinine" is from one to one and one-half hours, as compared with five to six hours for the same number of samples using inulin. The major difficulties in the inulin technique, namely, maintaining a constant plasma level and reliance on short urine collection periods, are obviated by its use. The chromogen plasma concentration remains fairly constant over a 24-hour period, and urine collection may be prolonged for several hours and thus reduce errors in urine collection at normal urine flows. The method thus lends itself to the study of the 24-hour variations in the filtration rate (29,

30). The procedure may be safely used in patients with heart failure, without danger of pulmonary edema induced by the administration of intravenous fluids.

The chromogen clearance is superior to the urea clearance in that it more closely approximates the filtration rate, the analytical procedure is simpler, and in our data the clearance apparently does not depend on the urine flow. We have not investigated the effects on the endogenous chromogen clearance of conditions in which there is excessive protein breakdown, such as may occur in association with malignant growths, starvation, hyperthermia, tuberculosis, hyperthyroidism, leukemia, muscular dystrophies, etc., nor has the effect of a high protein diet, which is known to raise the plasma creatinine level (30), been investigated.

The method is not applicable to the estimation of the filtration rate in infants, as is evidenced by the average endogenous chromogen/1.10 \times mannitol clearance ratio of 0.60. Mannitol/inulin clearance ratios in infants are the same as in adults, namely, averaging 0.90 (31). It is of interest in this connection that McCance (32) found that exogenous creatinine/inulin clearance ratios in infants are close to unity.

The plasma concentration of endogenous chromogen seems to have no definite relationship to the mechanism of excretion. In one subject with uremia in whom the plasma level of chromogen was as high as 9.9 mgm. per cent, the chromogen/inulin clearance ratio averaged 0.94, whereas in the subject in whom a ratio of 1.61 was observed (No. 12, Table II), the plasma concentration was only 1.2 mgm. per cent.

CONCLUSIONS

1. The endogenous "creatinine" chromogen/inulin clearance ratio has been determined in 94 and the endogenous chromogen/thiosulfate clearance ratio in 47 simultaneous clearance periods in 14 subjects without renal disease and with normal glomerular filtration rates. The respective ratios averaged 1.00 ± 0.018 with a range of 0.88 to 1.10, and 0.95 ± 0.018 with a range of 0.84 to 1.01.

2. The endogenous chromogen/inulin clearance ratio has been determined in 57 and the endogenous chromogen/thiosulfate clearance ratio in 51.

⁸ We are indebted to West, Chasis and Smith (28) for permitting us to use their data on the mannitol clearances in these infants.

simultaneous clearance periods in 13 subjects with reduced glomerular filtration rates due to renal disease. In 12 of the 13 subjects the respective ratios averaged 1.04 ± 0.109 with a range of 0.89 to 1.25, and 1.10 ± 0.292 with a range of 0.77 to 1.63. A discrepancy between the inulin and chromogen clearances of 10 per cent or greater appeared only in those cases with filtration rates below 40 cc./min. However, the absolute magnitude of differences between the two clearance values is of such a low order as to permit the chromogen clearance to be used as a clinical test of the glomerular filtration rate in the adult, even with marked impairment of renal function.

3. The sole marked discrepancy between the endogenous chromogen and inulin clearances occurred in a young female in the nephrotic stage of glomerulonephritis. In this instance the chromogen was excreted in a manner similar to exogenous creatinine. The chromogen/inulin clearance ratio averaged 1.61.

4. In infants the chromogen clearance is significantly lower than simultaneous mannitol clearances. The average chromogen/mannitol ratio, representing 24 periods, was 0.60 ± 0.066 with a range of 0.55 to 0.69.

5. Two grams of caronamide, orally, every four hours for eight doses to six subjects, and an infusion of PAH of sufficient concentration to maintain plasma levels of about 60 mgm. per cent in an additional six subjects, had no effect on the chromogen/inulin clearance ratio. However, caronamide in the above doses lowered the chromogen/inulin clearance ratio from 1.61 to 1.07 in the one subject exhibiting a marked discrepancy between the two clearances, providing additional evidence for tubular secretion of the chromogen in this subject.

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STUDIES ON AMINO ACID METABOLISM. II. BLOOD GLYCINE AND TOTAL AMINO ACIDS IN VARIOUS PATHOLOGICAL CONDITIONS, WITH OBSERVATIONS ON THE EFFECTS OF INTRAVENOUSLY ADMINISTERED GLYCINE¹

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INTRODUCTION

Glycine plays a role in the metabolism of serine (1), glutamic acid (2), glyoxylic acid (3), uric acid (4-6), lactate (6), creatine (7), glutathione (8), and the porphyrin of hemoglobin (9, 10). The amino acid has been administered, with inconclusive results, to patients with myasthenia gravis (11, 12), and with peripheral vascular disorders because of its vasodilating effects (13). Moreover, knowledge of the life span of erythrocytes (14) and of red cell destruction (15) has been advanced by using tagged glycine.

The normal concentration of glycine in blood, plasma, and erythrocytes has been reported (16) but nothing is known regarding its level in pathological conditions. The present data consist of glycine and total α amino N levels in patients with various diseases and following infusions of the amino acid.

METHODS

Glycine and total α amino N were measured by techniques previously described (17, 18). Each value is the average of duplicate determinations.

For the glycine tolerance tests, a sterile 10 per cent aqueous glycine solution (Eastman) was injected at 30 cc. per minute (total amount—1 gm. of the amino acid per 15 lbs. of body weight). In three subjects less glycine was administered. Blood glycine was determined before, and at intervals after, the injection. The urinary excretion of glycine during the experiments was also measured. Food was withheld for 12 hours prior to, and throughout, the test, but water was permitted *ad libitum*.

RESULTS

Normals: Based on previous data (16) and a few additional observations on normal subjects,

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the normal glycine range is now considered to be: for blood, 1.76-3.02 mgm. % (mean 2.10; S. D. 0.26); for plasma, 1.47-2.83 (mean 1.87; S. D. 0.34); for red cells, 1.64-3.73 (mean 2.49; S. D. 0.51). Any value for glycine or α amino N which deviated from the normal mean by an amount exceeding twice the standard deviation was considered significantly abnormal.³

Diseases of the liver and bile ducts: Five of 11 patients with liver disease had jaundice of hepatocellular origin; in two others the jaundice was either obstructive or hepatocellular. Subnormal blood and plasma glycine were found in one subject (subacute yellow atrophy [Table I, No. 10]) while in another (acute infectious hepatitis [No. 14]) plasma glycine was low whereas whole blood glycine was normal. Erythrocyte glycine was significantly elevated in five, contributing in four to a high whole blood glycine. These individuals had definite evidence of parenchymatous involvement.

Case P. L. with severe liver damage (Table I, Nos. 10-13) is of particular interest. Although determinations were not done until high protein feeding by stomach tube had already been initiated (with clinical improvement), blood and plasma glycine and total α amino N values were extremely low. As the patient continued to improve, plasma glycine and blood α amino N returned to normal while red cell glycine became abnormally high.

In two cases of liver disease, an intravenous glycine tolerance test was essentially normal.

Elevated red cell amino N was observed in three patients in one of whom plasma α amino N was also increased. No correlation between glycine, α amino N and protein concentration was evident.

³ In order to conserve space, only subjects who showed abnormal glycine values are recorded in Tables I and II. Should the reader desire additional data he may communicate with the authors.

Thus, except for low glycine and amino N in one case of subacute yellow atrophy, returning to normal with clinical improvement, no remarkable findings in plasma glycine were observed. Noticeable, however, was the tendency to elevated glycine and α amino N in the red cells.

Diseases of the kidney and essential hypertension: In two patients with acute glomerulonephritis the glycine values were normal, despite azotemia. Of nine subjects with chronic glomerulonephritis and renal insufficiency glycine concentrations were significantly increased in six and appeared unre-

TABLE I
Blood glycine and α amino N in various diseases

No.	Subject	Date		Amino N			Glycine			Ict. ind.	Total prot.	Alb.	Glob.
				Blood	Plasma	Erythrocytes	Blood	Plasma	Erythrocytes				
Diseases of the liver and bile ducts													
10	P. L.	2/26/46	Sub yel. atrophy	mgm. % 2.63	mgm. % 2.69	mgm. % 2.50	mgm. % 1.64	mgm. % 1.17	mgm. % 2.63	49	gm. % 5.41		
11	P. L.	3/2/46	Sub yel. atrophy	4.49	3.05	7.30	2.62	1.67	4.46	31			
13	P. L.	3/25/46	Sub yel. atrophy	4.88	3.30	7.45	3.03	2.26	4.29	15	7.07		
14	A. E.		Infect. hepatitis	5.13	3.42	10.20	2.10	1.35	4.36	17	5.90	2.45	3.54
16	L. S.	1/12/47	Infect. hepatitis	9.20	7.60	12.00	3.40	2.19	5.55	55			
18	L. S.	2/6/47	Infect. hepatitis	5.94	4.42	7.05	2.87	2.06	3.99	26			
24	J. R.		Cirrhosis; jaundice				3.07	1.98	4.43	34	6.25	2.78	3.47
28	R. H.		Chr. hepatit.; ? malignancy	3.92	2.78	6.24	2.95	2.33	4.21	6	8.00	3.32	5.08
Renal disease and essential hypertension													
33	N. S.		Chr. glomerul.; osteoporosis	4.52			3.56	3.64	3.25	45	6.63		
34	I. C.		Chr. glomerul.; uremia	6.03	5.14	8.58	3.08	3.04	3.19	75	6.70		
35	I. Z.		Chr. glomerul.; uremia hypert.	7.20	3.99	16.30	2.58	2.11	3.91	97	7.01		
36	J. Z.		Chr. glomerul.; uremia hypert.		3.86		2.96	2.96		166	7.01	3.99	3.02
39	M. K.		Hypert.; urem.; myocard. infarct.				2.24	1.72	3.57	150			
41	E. W.		Uremia; sulfadiazine kidney				2.48	2.95	1.34	96	5.24		
43	M. C.		Chr. glomerul.; nephrosis; uremia	4.72	3.14	10.30	3.53	3.00	5.40	50	4.04	1.82	2.22
44	J. M.		Chr. glomerul.; nephrosis; diab.				2.71	1.62	4.28	34	5.69	3.59	2.10
47	R. R.		Hyperten.; diabetes	5.02	3.86	7.09	2.69	2.31	3.36	35	6.10		
49	B. F.		Essential hypertension	5.81	4.80	7.07	2.65	2.06	3.39	37	7.89		
Endocrine diseases													
51	B. K.		Hyperthyr. and myopathy; untreated				1.54			BMR +40			
52	B. K.		Same; KI treatment				1.32	1.16	1.55	+24			
53	F. K.		Hyperthyr., KI treatment				2.59	1.71	3.57	+30	7.17		
54	M. S.		Hyperthyr. (postthyroidectomy) untreated	6.70	4.35	10.00	3.80	2.98	4.93	-25			
55	C. L.		Hypothy.; essent. hypertension				4.50	3.57		-16	6.22		
56	E. L.	1/6/47	Cretinism, untreated	8.03	4.41	14.70	3.55	2.78	4.97	-20	7.32	4.89	2.43
57	E. L.	1/8/47	Cretinism, untreated				3.59	3.27	4.17	-25			
58	M. H.	4/14/47	Acromegaly (preoperatively)				3.56	2.89	4.45	+20	6.76		
59	M. H.	4/25/47	Acromegaly (preoperatively)				3.46	3.01	4.10	+10			
60	M. H.	4/30/47	Acromegaly (postoperatively)				3.38	3.02	4.00				
61	M. H.	5/7/47	Acromegaly (postoperatively)				3.47	2.96	4.33	+ 3			
62	M. H.	5/28/47	Acromegaly (postoperatively)				3.40	2.84					
66	M. W.		Scleroderma and Addison's dis. (salt and Doca)	6.03	3.29	10.00	3.04	2.25	4.19	+ 4	6.09	3.48	2.63
67	E. A.	11/19/46	Malnutrit.; hypoprot.; hypometab.	4.38	2.16	8.98	1.90	1.40	2.93		3.65	1.27	2.38
68	E. A.	11/21/46	Malnutrit.; hypoprot.; hypometab.	4.69	2.69		4.70	3.30					
69	E. A.	12/26/46	Malnutrit.; hypoprot.; hypometab.	7.88	3.20		5.66	3.24		-29	3.86	1.12	2.74
70	E. A.	1/2/47	Malnutrit.; hypoprot.; hypometab.					3.51			4.00	2.29	1.71
71	E. A.	1/29/47	Malnutrit.; hypoprot.; hypometab.	5.70	3.39		5.12	3.04					
72	E. A.	2/2/47	Malnutrit.; hypoprot.; hypometab.				5.91	3.86			3.72	1.71	2.01

TABLE I—Continued

No.	Subject	Date		Amino N			Glycine			BMR	Total prot.	Alb.	Glob.						
				Blood	Plasma	Erythrocytes	Blood	Plasma	Erythrocytes										
Diseases of muscle																			
				mgm. %	mgm. %	mgm. %	mgm. %	mgm. %	mgm. %		gm. %	gm. %	gm. %						
74	A. L.	4/6/46	Myasth. grav.; testost. treat.	9.48	6.37	13.40	2.62	2.29	3.04	-24	7.38	5.08	2.30						
75	A. L.	6/25/46	Myasth. grav.; testost. treat.	8.45	6.05	11.00	3.03	2.56	3.51										
76	R. H.	12/15/45	Progress. musc. dystrophy	4.85	2.95		1.56												
77	R. H.	2/12/47	P.M.D.—after stilbest. treat.	5.55	3.36	8.81	2.61	1.83	3.75										
78	R. H.	2/24/47	P.M.D.—after stilbest. treat.				2.98	2.78	3.31										
79	R. H.	2/25/47	P.M.D.—after stilbest. treat.				2.91	2.06	4.27										
80	R. H.	3/11/47	P.M.D.—after stilbest. treat.				3.08	2.18	4.55										
Diseases of skin																			
81	E. G.		Pemphigus; treat. w/nirvanol	8.00	4.62	14.80	3.62	2.29	6.63	+11	6.43	2.63	3.03						
84	A. D.		Lupus erythem. dissem.				2.50	1.67	4.11										
86	E. C.		Urticaria				2.69	2.17	3.56										
Blood diseases																			
89	M. G.		Polycythemia vera; treated with urethane				3.76	1.19	5.48	+38	8.47	3.60	3.64						
90	B. M.		Polycythemia vera				3.51	2.38	4.13										
91	M. K.		Myelogen. leuk. and polycyth. vera				3.36	2.14	5.20										
92	J. F.	5/2/47	Chronic myelog. leukemia				3.21	2.14	5.35										
93	J. F.	5/23/47	Chronic myelog. leukemia treated with urethane				2.27	1.45											
Miscellaneous																			
94	G. F.		Rupt. interverteb. disc	4.62	2.95	7.41	2.62	2.08	3.78										
96	G. F.		Rupt. interverteb. disc				2.69	1.83	4.48										
99	O. K.		Bronchial asthma				2.79	1.98	3.68										
103	I. R.		Carc. pancreas; metast.				2.78	1.99	4.10										
106	M. Z.		Chr. ulc. colitis; malnut.				1.77	1.19	2.37										
107	A. B.		Chr. ulc. colitis; malnut.				1.56	1.61	1.41										
118	P. Y.	3/28/47	Rheumat. arthritis				1.37	1.11	1.91										
119	P. Y.	4/4/47	Rheumat. arthritis				1.65	0.96	3.27										
123	P. Y.	4/23/47	Rheumat. arthritis				1.85	1.04	3.88										
124	P. Y.	4/24/47	Rheumat. arthritis				1.98	1.10	4.05										
127	J. B.*		Rheumat. arthritis				2.86	1.72	4.80										
129	S. T.*		Rheumat. arthritis				1.69	1.15	2.67										
																	7.34	4.05	3.28

* We are indebted to Dr. Walter Bauer and Dr. Louis K. Dahl of the Massachusetts General Hospital, Boston, for generously providing these patients for our study.

lated to the degree of nitrogen retention and to α amino N which, except in two cases, was normal. The glycine tolerance test was not remarkable in two subjects with chronic nephritis, one with marked hypoproteinemia.

Endocrine disease: Five patients with hypermetabolism were studied. In one (No. 51) who showed myopathy simulating myasthenia gravis, all the glycine values were low. In another subject with acromegaly (Nos. 58-62) the values

were elevated; this persisted for at least one month after ablation of the hypophysis. Blood amino N was normal in those subjects in whom it was determined.

In five cases of hypometabolism, glycine tended to be elevated, whereas in only one, a cretin (No. 56), was the α amino N also increased. In one patient with hypometabolism and nutritional hypoproteinemia plasma amino N was originally low, then steadily rose, while plasma glycine values

TABLE II
Intravenous glycine tolerance test

No.	Init.	Sex	Diagnosis	Wt.	Glycine given	Time after gly.	Blood glycine			Glycine excret.
							Blood	Plasma	Cells	
				lbs.	gms.	min.	mgm. %	mgm. %	mgm. %	mgm.
5	C. L.	F	Essent. hyperten. hypothyroid.	210	14	0	4.50	3.57	6.08	277
						20	26.7	32.2	17.4	
						80	12.7	12.8	12.4	
						140	9.99	9.20	11.4	
						260	7.87	7.27	8.89	
6	M. H.	F	Acromegaly (one week postoperatively)	150	9	0	3.42	2.96	4.33	614
						20	15.9	20.7	7.82	
						80	9.81	11.2	7.52	
						200	6.79	6.23	7.74	
7	E. A.	F	Nutrit. hypoprot.; hypometabolism	76	5	0		3.51		34
						20		32.6		
						80		11.1		
						140		7.49		
10	P. Y.	M	Rheumatoid arthritis	121	8	0	1.87	1.28	3.25	
						20	5.88	5.54	6.07	
						80	2.96	2.14	4.91	
						200	2.61	1.36	5.58	
11	P. Y.	M	Rheumatoid arthritis, 30 gms. glycine orally 12 hours before test	121	8	0	1.98	1.10	4.05	381
						20	6.34	6.15	6.10	
						80	3.03	2.13	5.28	
						200	2.13	1.27	4.30	

increased suddenly from low normal and remained elevated. The changes could not be related to therapy.

The glycine tolerance test of two patients with endocrine disturbances, one (Table II, No. 5) with hypothyroidism and the other (Table II, No. 6) with acromegaly and hypermetabolism, showed very high plasma and red cell glycine following injection, with an abnormally slow decline thereafter. Fasting blood glycine was also abnormally high. In Case No. 7 (hypometabolism and nutritional hypoproteinemia) plasma glycine rose markedly after injection. The subsequent decline, however, was essentially normal.

Two diabetics, both insulin treated, showed low normal plasma glycine.

Elevated whole blood and red cell glycine was observed in a case of Addison's disease with scleroderma.

Diseases of muscle: Four patients with myopathies were studied. One of two cases of myasthenia gravis showed normal blood glycine and α amino N; he was receiving prostigmine therapy. In the other individual (No. 74), who was largely

bedridden, glycine and α amino N were elevated. She had been taking methyltestosterone orally for nine months. Unfortunately these subjects were not studied prior to treatment.

One subject with progressive muscular dystrophy and hypometabolism (No. 76) showed low normal amino N and glycine levels one and a half years ago. After stilbestrol therapy for several months, his blood glycine became elevated. Glycine tolerance, however, was normal.

Patient B. K. (No. 51), with myopathy and hyperthyroidism, discussed before, showed low blood and plasma glycine. An oral glycine tolerance curve, reported in a previous communication (16), was abnormally flat.

Rheumatoid arthritis: This group comprised six cases, all with active disease. One patient (No. 118) had moderate anemia and spiking fever. Low plasma glycine was observed in two (Nos. 118, 129) one of whom (No. 118; Table II, Nos. 10, 11) showed remarkably abnormal glycine tolerance curves. Not only were they relatively flat (Figures 1 and 2) but also they returned practically to the pre-injection values, in contrast to the

other subjects. Furthermore, glycine administered the night before the test did not affect the pattern of response. The urinary glycine of this patient during the tolerance test was not significantly different from that of other individuals. Administration of glycine (10 gms. a day for four days orally, 30 gms. orally or 8 gms. intravenously) did not influence his creatinine or creatine excretion.

Ten minutes following the first glycine injection, the patient suddenly experienced remarkable "loosening" of the joints with painless mobility for the first time in two weeks. After eight hours, the pain and limitation of motion returned gradually. These observations could not be repeated.

Blood diseases: Two subjects with erythremia had elevated erythrocyte glycine; in one, receiving

urethane, the plasma value was low. Elevated red cell glycine was also found in two patients with chronic myelogenous leukemia. In one (No. 92) the concentration decreased in both blood and plasma after the leucocytes had dropped from 230,000 to 12,900/mm. following treatment with urethane.³

One patient with untreated pernicious anemia showed normal glycine values.

Miscellaneous: High red cell glycine and amino N were observed in a case of pemphigus vulgaris (No. 81) (under nirvanol treatment); red cell glycine was also elevated in disseminated lupus and urticaria. Significantly low values were observed in two cases of ulcerative colitis with malnutrition (Nos. 106, 107). In one (No. 106) plasma glycine was low whereas the level in whole blood was normal. In the other subject (No. 107) blood and red cell glycine were low but the concentration in the plasma was normal. High red cell glycine levels were observed in a case of ruptured intervertebral disc (No. 94), and in one patient with carcinoma of the pancreas (No. 103).

Effects of intravenously administered glycine

No untoward reactions occurred following intravenous glycine. Within ten minutes the subjects generally experienced warmth and flushing of the face, and sometimes paresthesiae in fingers and toes. Because glycine is said to affect the kidneys of animals adversely (19), the urine was examined in all subjects during and after the test, but no changes were observed.

Following glycine infusion, blood values changed rather uniformly (Figure 1 is typical). Twenty minutes after injection plasma and red cell glycine became markedly elevated, the former to a greater extent than the latter. Subsequently plasma glycine declined gradually, sometimes falling to lower values than whole blood glycine since red cell glycine lagged behind plasma changes and, in some cases, remained elevated (Table II, No. 10). In only one subject (Table II, Nos. 10, 11) did plasma glycine return to the pre-injection level during the period of observation.

The height of the 20-minute rise in blood and plasma glycine seemed to be related to the fasting values; this relationship was not apparent for erythrocyte glycine.

INTRAVENOUS GLYCINE

TOLERANCE TEST

A.S., BRONCHIECTASIS
BODY WEIGHT 168 LBS.

INJECTED 11 GRAMS OF GLYCINE
IN 110 CC DIST. WATER AT TIME 0

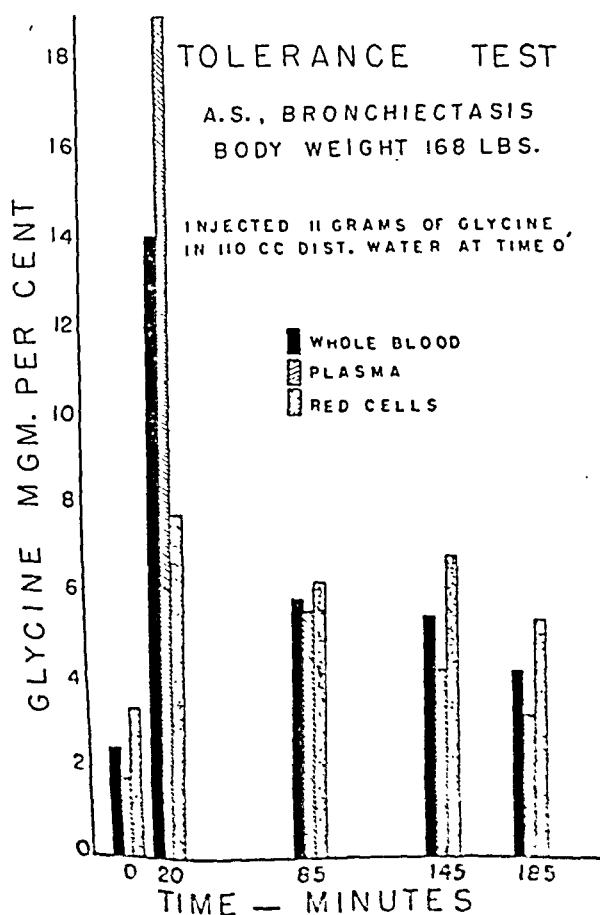


FIG. 1. EFFECTS OF INTRAVENOUSLY ADMINISTERED GLYCINE

CASE OF P.Y., RHEUMATOID ARTHRITIS

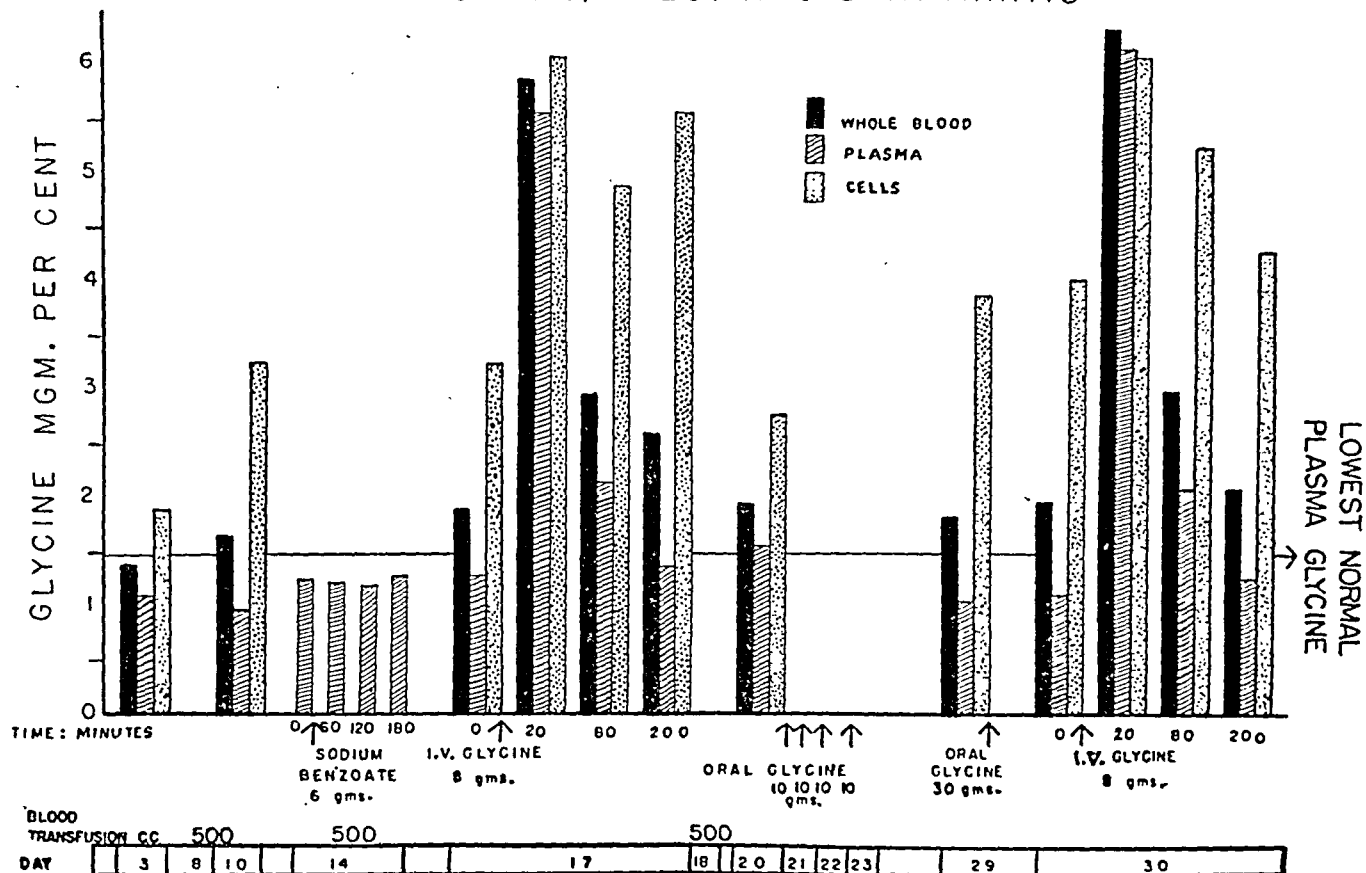


FIG. 2. INTRAVENOUS GLYCINE TOLERANCE TEST

Total glycine excreted during the test, always less than a gram, varied widely and was unrelated to the blood glycine curve.

The slow rise in erythrocyte glycine compared with plasma glycine may be due either to its slow diffusion into red cells or to its early transformation or conjugation within the cells. Experiments exploring these possibilities are presented below.

Oxalated blood, divided into four samples, was treated as follows:

Sample 1: 24 cc. were mixed with 0.5 cc. of an aqueous glycine solution (1.16 gms. per 100 cc.). The mixture was immediately analyzed for blood, "plasma," and red cell glycine.

Sample 2: 24 cc. were mixed with 0.5 cc. physiological saline; an aliquot was similarly analyzed.

Sample 3: to 12.8 cc. of plasma was added 0.5 cc. glycine solution; glycine was determined immediately.

Sample 4: to 12.8 cc. plasma was added 0.5 cc. isotonic sodium chloride solution and the mixture was analyzed.

Determinations were repeated two and four hours later on samples of the four mixtures which were incubated at 37° C. with frequent shaking. The results (Table III) show that in *Sample 2*

the glycine of blood, plasma and erythrocytes rose slowly during incubation. The highest increase occurred in the cells where, it seemed, some glycine was either liberated from a conjugated form or was otherwise evolved. In *Sample 1* the artificially augmented plasma glycine declined slightly while cell glycine increased considerably. The total glycine of the mixture, however, rose only by an amount roughly equal to that observed in *Sample 2*.

TABLE III

Changes in plasma and red cell glycine in vitro

Hematocrit was 45 per cent packed cells and it remained constant. All values expressed in mgm. per cent. Incubated at 37° C.

In-cubation time	Sample 1 Blood + glycine			Sample 2 Blood + saline			Sample 3 Plasma + glycine	Sample 4 Plasma + saline
	Blood	Plasma	Cells	Blood	Plasma	Cells		
hrs.								
0	25.2	41.9	4.69	2.17	1.75	2.68	41.7	1.91
2	25.6	40.2	7.71	3.08	1.99	4.41	41.9	1.72
4	27.2	38.6	13.20	3.57	2.34	5.09	43.4	1.94

DISCUSSION

The relatively normal plasma glycine concentrations in acute infectious hepatitis and cirrhosis of the liver are in accord with the normal total amino acid levels reported in these diseases (20-24). Only in complete hepatectomy (25) or severe liver damage (20) as in acute yellow atrophy (26-28) are amino acid values increased. The low glycine found in our case of subacute yellow atrophy might be referable to inability to synthesize this "non-essential" amino acid or to malnutrition, as suggested by the low plasma glycine observed in chronic ulcerative colitis with malnutrition. However, blood amino acid concentrations in experimental nutritional hypoproteinemia are normal (29) while our case of nutritional hypoproteinemia (Table I, Nos. 67-71) showed exceptionally high blood and plasma glycine levels after a low initial value.

The normal glycine tolerance in liver disease also is in agreement with the experience of others who found amino acid-loading tests useless in the diagnosis of liver disease (20, 23).

The high glycine concentrations in chronic glomerulonephritis is not related to the hypoproteinemia, the hyperaminoacidemia (30, 31) or the nitrogen retention found in kidney disease. Nor can the glycinemia be explained by inadequate urinary glycine excretion since the amount thus eliminated is usually small. It is possible that the metabolism of glycine in the diseased kidney is disturbed in view of the function of this organ in elaborating guanidoacetic acid from glycine and arginine (7, 32). The normal glycine tolerance in two nephritics, one with the nephrotic syndrome, is in accord with the reported undisturbed utilization of glycine in nephrosis (33).

The literature on blood amino acids in endocrine disturbances is extensive (34, 35). Thyroidectomy or thiouracil lowers blood amino acids in rats whereas tissue amino acids are unaffected. Okada and Hayashi (36), on the other hand, found no such alterations following thyroidectomy.

Our observations indicate that hypometabolism tends to be associated with high blood glycine and normal α amino N. The abnormally elevated plasma amino acid curve reported by Witts (21) after glycine administration in a patient with myxedema, is in accord with our results with the gly-

cine tolerance test in hypometabolism. Presumably the tissues of these cases with high fasting plasma glycine are also rich in glycine and accordingly are less able to take up additional amounts of the amino acid.

Contrary to what might be expected from the above, glycine values were generally normal in hypermetabolism. This is in agreement with the blood amino N findings of Maddock *et al.* (37) but is in contrast to the elevated values reported for the plasma, liver, and skeletal muscles of rats treated with thyroxine (35). The low glycine and low oral glycine tolerance test (16) in one case of hyperthyroidism with severe myopathy and creatinuria may be related to this complication.

The elevated glycine in the acromegalic may be referable to the anabolic effect of the growth-promoting hormone of the pituitary on protein metabolism although this hormone is said to lower the blood amino acids (38-40).

The high glycine observed in a case of Addison's disease with hypotension and hypochloremia despite DOCA and salt therapy is noteworthy in view of decreased plasma amino acids in adrenalectomized rats, which increased markedly after administration of cortical extract (35).

The relation between glycine and creatine and the role of the latter in the metabolism of muscle have been studied extensively (7, 11, 12, 41-55). It is likely that in the myopathy of thyrotoxicosis (44) and in myopathies in general (34) there is no impairment of creatine synthesis, but rather of creatine storage and utilization. This concept is compatible with the low glycine values obtained in the patient with thyrotoxicosis and myopathy and in the subject with progressive muscular dystrophy if we assume that excessive creatinuria serves to deplete glycine stores faster than the amino acid can be synthesized in the body.

The elevated glycine and total blood amino acids in one patient with myasthenia gravis who was receiving methyltestosterone may be referable to the anabolic effects of this hormone (56).

Little which is non-speculative can be said concerning the strikingly low glycine in certain cases of rheumatoid arthritis. It is noteworthy that the glycine content of elastin, an important protein constituent of ligaments and tendons, is 25.5 per cent (57). The low tolerance curve and rapid clearance of injected glycine from the plasma of

one subject suggest an undue avidity of the tissues for this amino acid or its rapid destruction. The latter seems more likely since large amounts of glycine administered twelve hours prior to the test had no effect on the tolerance. In addition a hippuric acid test, to be reported elsewhere (58), revealed that hippuric acid excretion following ingestion of benzoate was subnormal in this patient. Further investigation of the role of glycine in this disease is in progress.

The high erythrocyte glycine in both polycythemia vera and myelogenous leukemia was striking. The decrease in glycine in myelogenous leukemia consequent to urethane therapy and the strikingly low plasma glycine in the polycythemic, who, under treatment with the same chemical, showed at the same time a significantly high erythrocyte glycine warrant further study of glycine metabolism and the effects of urethane in those blood dyscrasias.

Following the intravenous administration of 1 gm. of glycine per 15 lbs. of body weight plasma glycine rises to between 14 and 38 mgm. per cent. Assuming a plasma volume of 3.5 liters in patient A. S. the injection of 11 gms. of the amino acid should theoretically have resulted in a plasma glycine of about 300 mgm. per cent if all the glycine had remained in the plasma. After 20 minutes, only about 7 per cent of the injected amino acid could be accounted for in this patient's blood. These discrepancies in view of the relatively insignificant urinary glycine, indicate a rapid uptake of the amino acid by the tissues. Similar observations, based upon total amino N determination, have been made after administration of glycine or of amino acid mixtures (10-13, 59). In this respect it is significant that the amino acid levels in muscles and kidney remain high for more than three hours after glycine injection (60), whereas liver amino acid (60) and blood glycine had already returned almost to normal. Furthermore, in our experiments with benzoate-glycine conjugation (58), we observed that the ingestion of 10 gms. of glycine 12 hours prior to benzoate administration increases the amount of hippuric acid formed, despite the fact that plasma glycine is normal at the time of the test. The supplementary glycine probably was present in the tissues, either free or bound in an easily available form.

After the ingestion of protein plasma and erythro-

cyte amino acids rise, the latter lagging behind the former and persisting for several hours even when plasma amino acid was returning toward normal (61). Similar observations regarding glycine have been described after oral administration of this amino acid (16, 62). The same results are obtained following intravenous glycine. On the basis of our *in vitro* experiments this lag is best interpreted as being due to a slow diffusion of glycine into and out of the erythrocytes.

SUMMARY

Blood glycine and α amino N have been studied in various pathological states. Low blood glycine was observed in one case of subacute yellow atrophy, one subject with hyperthyroidism with myopathy, and in two cases of ulcerative colitis. The lowest values were observed in subjects with rheumatoid arthritis.

A distinct tendency toward elevated glycine, particularly in the erythrocytes, was found in some patients with liver disease, with chronic glomerulonephritis, with hypometabolism, with polycythemia vera, and with leukemia.

No relation was evident between the concentrations of glycine, total α nitrogen, plasma protein, and blood non-protein nitrogen.

The intravenous administration of glycine results in a rapid rise and subsequent decline in both plasma and red cell glycine concentration. The latter lags somewhat behind the former, which, on the basis of *in vitro* studies, is interpreted as being due to slow diffusion of glycine into and out of the erythrocyte.

Certain abnormalities in the glycine tolerance curve were observed in a few pathological subjects.

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STUDIES ON AMINO ACID METABOLISM. III. PLASMA GLYCINE CONCENTRATION AND HIPPURIC ACID FORMATION FOLLOWING THE INGESTION OF BENZOATE¹

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The conjugation of benzoic acid with glycine, resulting in the formation of hippuric acid, takes place in liver (1) and kidney (2). The urinary output of this conjugate after the administration of benzoate is used to measure this aspect of hepatic function (3).

Theoretically, decreased hippuric acid formation may be due to hepatic and/or renal disease or to an inadequate supply of glycine. In this regard it is not clear to what extent this amino acid is available as such in the liver or is formed *ad hoc*, either in this organ or elsewhere. The present investigation was undertaken in the hope that data bearing on this question might be obtained.

PROCEDURE AND METHODS

Six or 10 gms. of sodium benzoate² were given orally to fasting normal or diseased subjects, who were induced to drink approximately 500 cc. of water. The urine collected during the subsequent three hours was analyzed for its hippuric acid content according to the method of Quick (5). Fasting venous blood was obtained just before the ingestion of benzoate and at intervals thereafter. In some cases an additional fasting sample was taken one-half or one hour before the administration of benzoate. Plasma glycine was determined by the method of Alexander *et al.* (6). The influence of orally administered glycine was also studied in a few cases.

RESULTS

Benzoate was administered without additional glycine 11 times to ten individuals (Table I), and in all instances except one (No. 11) a significant decrease in plasma glycine occurred. In some cases the decline began within $\frac{3}{4}$ hour after ingestion and continued thereafter (Nos. 1, 2, 7, 8, 9). In others it remained at the low level reached

within one hour (Nos. 3, 4, 10); in two subjects (Nos. 5, 6) it started to rise again during the period of observation. The largest drop in plasma glycine amounted to 2.04 mgm. per cent (52 per cent of the initial concentration); in general, however, the extent of the drop was between 20 and 32 per cent of the fasting glycine concentration, and was unrelated to this value.

The amounts of hippuric acid excreted varied widely. It is seen that in the only case in which no decrease in plasma glycine occurred, the hippuric acid excretion was definitely subnormal (1.92 gms., as compared with a minimal normal excretion of 3 gms.) It appears, however, from a comparison of cases 9 and 11, and 2 and 5, that no positive correlation exists between the quantity of excreted hippuric acid and the decrease in plasma glycine, although admittedly the number of observations is too limited to permit drawing definite conclusions.

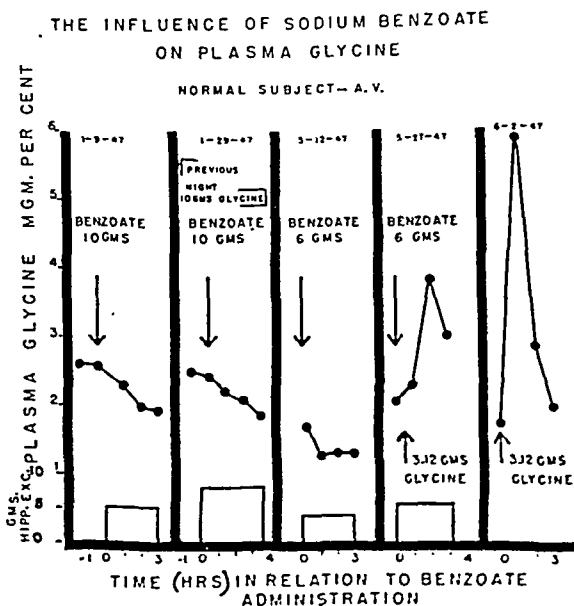


FIG. 1.

¹ Supported by a grant from the John and Mary Markle Foundation, New York City.

² Sodium benzoate was used in preference to benzoic acid because of its more rapid absorption from the intestinal tract (4).

Five experiments were performed on one normal subject (Nos. 2, 3, 12, 13, 13a). The data are presented in Figure 1. The ingestion of 10 gms. of benzoate resulted in a larger hippuric acid excretion and a greater absolute drop in plasma glycine than the administration of 6 gms. Fur-

thermore, the ingestion of 10 gms. of glycine the night prior to the test resulted in a larger hippuric acid excretion, without, however, a greater decline in plasma glycine than obtained following the administration of 10 gms. of benzoate alone. The administration of glycine half an hour after the

TABLE I
The effect of benzoate on plasma glycine and on hippuric acid formation

	No.	Sub.	Sex	Age	Diagnosis	Na benz. taken	Hipp. acid excretion		Urine vol.
No supplementary glycine						<i>gms.</i>	<i>gms.</i>	<i>hrs.</i>	<i>cc.</i>
	1	M. G.	F	21	Bronchial asthma	10			
	2	A. V.	M	35	Normal (1-9-47)	10	5.10	3	440
	3	A. V.	M	35	Normal (5-12-47)	6	3.77	3	360
	4	F. B.	F	44	Essential hypertension	10			
	5	W. T.	M	60	Arterioscler.; heart disease, congestive failure	6	3.48	3	355
	6	L. S.	M	70	Infectious hepatitis	6	4.15	3	950
	7	I. G.	M	43	Infectious hepatitis	6	4.28	3	310
	8	S. R.	M	49	Car. of pancreas	6	4.2	3	300
	9	E. A.	F	32	Nutrit. hypoproteinemia	6	2.03	4	200
	10	Th. W.	M	52	Virus pneumonia; malnutrition. Recon- valescent (5-8-47)	6	2.61	3	230
11	P. J.	M	25	Rheumatoid arthritis	6	1.92	3	380	
Additional glycine given*	12	A. V.	M	35	Normal (1-29-47); 10 gms. glycine taken night before test	10	7.69	3½	300
	13	A. V.	M	35	Normal (5-27-47); 3.12 gms. glycine taken ½ hour after ingestion of 6 gms. Na benzoate	6	5.18	3½	445
	13a	A. V.	M	35	Normal (6-2-47); 3.12 gms. glycine w/o Na benzoate				
	14	Th. W.	M	52	Virus pneumonia; malnutrit. Reconva- lescent. 3.12 gms. glycine taken simul- taneously w/ 6 gms. Na benzoate	6	4.92	3	640

TABLE I—Continued

	No.	Sub.	Plasma glycine—mgm. per cent Fasting					Plasma glycine—mgm. per cent After benzoate ingestion													Plasma glycine decrease	
			Time in minutes					Time in minutes														
			-60	-45	-30	-15	0	15	30	45	60	75	90	105	120	135	150	165	180	195	mgm. per cent	per cent
No supplementary glycine	1	M. G.		2.66			2.47			1.82				1.65							0.82	33
	2	A. V.	2.61				2.56		2.27				1.99			1.91					0.65	21
	3	A. V.					1.65				1.27							1.30			0.35	21
	4	F. B.					2.06			1.56				1.47			1.48				0.58	28
	5	W. T.					2.10				1.37				1.15						0.68	32
	6	L. S.	2.19				2.17			1.70								1.42			0.26	12
	7	I. G.			1.91		1.99				1.61				1.61			1.41			0.58	29
	8	S. R.		1.99			1.85		1.75						1.45						0.40	22
	9	E. A.			3.86		3.90		2.81			0.92			2.17						2.04	52
	10	Th. W.		1.44			1.33					0.88			0.88			0.84			0.49	37
	11	P. J.		1.26			1.29					1.22			1.20			1.28			0	0
Additional glycine given*	12	A. V.		2.49			2.41				2.19				2.10				1.82		0.59	24
	13	A. V.					2.02		†		2.31				3.80				2.99			
	13a	A. V.					1.69†		5.84				2.78				1.91					
	14	Th. W.					1.73†				2.89				2.60				2.00			

* Glycine administered orally.

† Time of glycine ingestion.

benzoate ingestion in an amount equivalent (chemically) to the benzoate ingested also increased the hippuric acid output; plasma glycine rose, but to a lesser extent than when glycine alone was administered.

The influence of additional glycine on hippuric acid formation was also apparent in subject Th. W. (compare No. 10 with No. 14 of the table).

DISCUSSION

Leuthardt and Glasson (7) and Quick (1) assumed that the glycine needed for the conjugation of benzoic acid is synthesized by the liver at a rate which is the principal limiting factor in hippuric acid synthesis. It has been known (1, 8-11) and reconfirmed above that the administration of glycine in addition to benzoate increases hippuric acid formation. In senile individuals, subnormal hippuric acid formation may be due to inability of the liver to furnish enough glycine since supplements of this amino acid restore hippurate excretion to normal (12).

That stores of free glycine are present in the body has been shown (13, 14). The drop in plasma glycine occurring after benzoate administration, first reported by Christensen (14) and confirmed by us, indicates that these stores are utilized in hippuric acid formation probably faster than the amino acid can be synthesized or mobilized. The question arises if and to what extent the concentration of plasma glycine influences the synthesis of hippuric acid. The subnormal hippuric acid output and absence of plasma glycine drop in Case No. 11, whose fasting plasma glycine was extremely low, can be interpreted in two ways: either not enough glycine, as reflected by the low plasma glycine concentration, was available for hippuric acid formation, or a primary disturbance in liver function of this patient prevented glycine-benzoate conjugation. In accord with the latter concept is the decline of plasma glycine in Case No. 10, whose fasting plasma glycine was also subnormal.

It appears that an elevated blood glycine concentration consequent to glycine ingestion (compare No. 3 with 13, 11, and with 14) is accompanied by increased hippuric acid formation. A high plasma glycine level per se, however, does not necessarily result in a very large hippuric acid output even though a considerable drop in plasma

glycine may occur after benzoate ingestion (No. 9). It would thus seem that the concentration of plasma glycine cannot be considered an absolute measure of the total amount of glycine available for uptake by the liver (and kidney?). The net decrease in plasma glycine following benzoate administration may be influenced not only by uptake by the liver but also by other factors such as plasma volume, and by the rate of replacement of plasma glycine by tissue stores of the amino acid. In this respect it is noteworthy that the subject of Experiment 9 had an exceptionally low body weight and, accordingly probably a low plasma volume. Furthermore, an intravenous glycine tolerance test on this subject (reported elsewhere [15]) indicated a slow equilibration of plasma glycine with the tissues. It should be pointed out, however, that because of the low body weight 6 gms. of benzoate in this patient would be comparable to a much larger dose in heavier subjects.

That glycine given simultaneously with benzoate is utilized for hippuric acid synthesis is indicated not only by increased hippuric acid formation but also by a smaller rise in plasma glycine than when the same amount of amino acid is given alone. On the other hand, the rise which did occur, even when glycine was simultaneously administered in amounts *chemically equivalent* to the ingested benzoate, indicates that part of the ingested glycine was not utilized during the observation period. This is probably attributable to a faster rate of glycine absorption than benzoic-glycine conjugation, whatever the limiting factors of the latter may be.

The large hippurate excretion observed when glycine was taken the night prior to benzoate suggests that glycine was stored in the liver (and kidney?) either in free, or in otherwise readily available form. The latter is more probable since it is known that three hours after glycine injection the concentration of easily extractable amino acids in the liver has returned to normal (16).

From the data it appears that within certain limits the rate of conjugation of benzoate with glycine depends to some extent upon both the dose of benzoate and the availability of glycine in the body. Under certain conditions the latter may be significantly curtailed. Accordingly, to obviate these factors in hippuric acid studies of liver function, it might be advantageous to administer

the benzoate in doses determined by the total body weight or surface area and to give the amino acid before or with the benzoate in at least a chemically equivalent amount.

CONCLUSIONS

1. In ten out of 11 experiments the administration of sodium benzoate resulted in a decrease in plasma glycine concentration. In one case with subnormal hippuric acid excretion the plasma glycine remained unaltered.

2. The administration of glycine before or together with the benzoate results in a larger output of hippuric acid than when benzoate alone is ingested.

3. The rate of conjugation of glycine to benzoate, the availability of glycine for this purpose, and the size of the dose of benzoate, are important factors which affect hippuric acid synthesis in man.

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THE AURICULOTEMPORAL SYNDROME A CLINICAL AND PHARMACOLOGIC STUDY

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The auriculotemporal syndrome which develops after injury to the parotid gland, consists of flushing and profuse sweating, upon eating, over the cutaneous distribution of the auriculotemporal nerve on the injured side. Although there have been few cases reported since the original description by Frey (1) in 1923, the syndrome is not rare, having been noted repeatedly, for example, in clinics observing patients after operation on a parotid gland (2, 3). The syndrome is of interest to the physiologist because it affords an opportunity to study the consequences of denervation of cholinergic end organs. It is our purpose to present three instances of this syndrome with some observations relating to its mechanism.

CASE HISTORIES

Case 1: J. B., a 68 year old man, BIH No. 78777, entered the hospital on 9/29/44 complaining of transient weakness and dysarthria. These symptoms were believed to be consequent to a recent cerebrovascular accident.

Forty-one years ago, while in the Russian army, the patient had had typhoid fever, complicated by right parotitis. Two attempts to drain the parotid gland by vertical incisions were unsuccessful. Two incisions, 2 cm. lateral to the right upper border of the thyroid gland, resulted in drainage for approximately 50 days. *Approximately one month after the successful drainage, and two months after the onset of the parotitis, the patient noticed the development of profuse sweating over the right side of the face while eating. Although ingestion of many foods was followed by sweating, the phenomenon was particularly marked when apples were eaten. On each occasion just prior to the onset of sweating the patient experienced a sensation of warmth over the involved area. Redness, swelling and pain, however, were absent; at no time was numbness noted over the involved area. The amount of sweating had become less pronounced during the previous three years.*

On physical examination, scars on the face and neck were noted. The opening of the right Stenson's duct was protuberant; saliva could not be expressed from it. The orifice of the left duct appeared normal and saliva was easily expressed. Perception of heat and cold was normal and equal on both sides of the face. There was cutaneous hypaesthesia in the distribution of the right auriculo-

temporal nerve. There was a right facial weakness and ptosis of the right upper eyelid; the latter was said to have been present since the patient's recent cerebral vascular accident. The cranial nerves were otherwise normal.

Case 2: T. H., a 63 year old man, MGH No. 484270, with a history of recent sinusitis, had had bilateral suppurative parotitis, of unknown etiology, twenty-five years previously; this had been drained through incisions over the glands on both sides. *Several weeks after operation the patient noted the occurrence of profuse sweating and a feeling of warmth on the left side of his face when eating. The phenomenon had continued until admission. It was brought on by eating any food, especially apples. At no time had the patient noticed any numbness or paralysis of his face. Physical examination was not remarkable except for the old drainage scars over both parotid glands. The parotid glands were normal to palpation and saliva was easily expressed from both ducts. Sensation over the face was normal and the function of other cranial nerves was apparently intact.*

Case 3: D. E. S., a 49 year old married woman (referred by Dr. I. T. Nathanson), had had a tumor of the right parotid gland removed in 1942. *Approximately one year later she noted the onset of sweating following the eating of various foods. The sweating involved the right side of the face in the region of the incision. Rarely a sense of heat was noted but visible flushing was never observed. No specific food was noted to be particularly effective in producing the sweating reaction. Although on rare occasions a whole meal did not produce sweating, hot tea by itself occasionally produced profuse perspiration. She had had mumps as a child, but with no sequelae.*

OBSERVATIONS

Observations in Case 1: The observations on sweating were made by means of the Minor starch-iodine test (4) which consists of applying a solution of iodine (15 cc. 1 per cent iodine, 5 cc. of castor oil and 80 cc. 95 per cent alcohol) and after drying, lightly dusting the skin with starch and noting the onset of sweating which is signalized as black spots. Ten seconds after taking a bite of apple and while chewing, the patient noticed a feeling of warmth over the right face, and a slight flush was noted on this side. Five seconds later, sweating appeared in part of the area in-

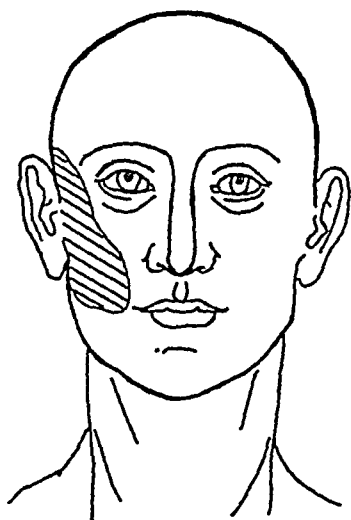


FIG. 1. THE DISTRIBUTION OF SWEATING OBSERVED IN CASE 1, REPRESENTED BY THE CROSS-HATCHED AREA

cluded in the distribution of the auriculotemporal nerve (Figure 1). The sweating could be induced by chewing any food, but was absent or slight when the patient chewed paraffin. The application of ice to the right face before chewing food resulted in a decreased amount of sweating in the chilled area. Sweating was absent when the patient held but did not chew food placed in his mouth. When the tongue was swabbed with vinegar, a small amount of sweating appeared. The response was independent of the region of the tongue swabbed. When the patient chewed a pledget of cotton soaked in vinegar, the amount of sweating, as judged by the extent and depth of the starch-iodine reaction, was more marked. Psychic salivation and sweating could not be induced. Sweating induced by the general application of heat to the body was irregularly diminished over the right side of the face.

Procainization of the right auriculotemporal nerve resulted in anesthesia over part of the distribution of the nerve. In the area of anesthesia sweating failed to appear after the usual stimulation. Novocaine injection¹ of the right superior cervical ganglion resulted in a Horner's syndrome, suffusion of the conjunctiva, increased warmth, and dilation of the right hand veins. While the cervical sympathetics were blocked, eating an apple induced sweating in 45 seconds and 20 seconds in two successive trials. The patient was apprehen-

sive during the whole period of injection and for a short time afterward.

A series of experiments was performed to determine whether there was hypersensitivity of the sweating mechanism in the involved area. In normal controls, local sweating was observed over and around intradermal wheals made by the injection of 0.1 cc. of various concentrations of acetylcholine bromide dissolved in isotonic saline solution. By using increasing dilutions, a measure of the sensitivity of the local sweating apparatus to acetylcholine might be obtained. While sensitivity was found to vary in different individuals and in different regions of the body, the end points were consistent. In this patient, intradermal wheals in the involved area on the right side of the face produced with 0.1 cc. of isotonic saline solution containing 0.0001–0.1 gamma of acetylcholine bromide resulted in local sweating. Similar concentrations of acetylcholine in symmetrical positions on the left face of the patient did not produce sweating. A wheal on the left side of the face which contained 0.5 gamma acetylcholine per 0.1 cc. produced sweating.

Iontophoresis (3.4 milliamperes for ten minutes through a 1 sq. cm. pad) of a 1:12,500 solution of acetylcholine bromide in isotonic saline solution, produced sweating on the involved right side of the face and no sweating on the uninvolved left side on two attempts, with no sweating on either side on the third attempt. Ten mgm. of acetylcholine bromide injected subcutaneously did not result in facial sweating.

The possibility was examined that disturbances in vasomotor nervous function are present in the auriculotemporal syndrome. The variation in skin temperature of the involved areas in response to cooling and heating the body and extremities was measured and compared to symmetrical uninvolved areas. To determine the time of occurrence of the maximal response to heating and cooling, measurements of the skin temperature of a finger tip were made. The measurements of skin temperature were made at frequent intervals with a thermocouple. The experiment was performed in a constant temperature room, 20° C. The patient was seated and the legs, arms and chest were uncovered. Within 20 minutes a distinct fall in finger tip temperature was observed (Figure 2) which reached a maximum in 70 minutes. The

¹ The authors are indebted to Dr. Reginald Smithwick for the procaine blocks in Cases 1 and 2.

right ear skin temperature fell from 29.5°C to 25.5°C in this interval of time. At the arrow (70 minutes) one leg and one arm were immersed in running warm water ($T\ 42^{\circ}$); the patient was covered with blankets and hot water bottles were applied to the chest and back. Approximately 15 minutes later, a distinct and sustained rise in finger and left ear temperature occurred. The involved right ear, however, showed no rise in temperature in response to heating (Figure 2); furthermore, the increase in right facial skin temperature in response to heat was less than that observed in a symmetrical point on the uninvolved left side.

An attempt was made to measure and compare the secretion from the parotid glands in this patient. No saliva could be collected from the right parotid duct. Secretion from the left duct appeared normal.

Observations in Case 2: In this subject flushing was observed over the left side of the face ten seconds after he began to eat an apple, and in 15 seconds sweating was observed over the entire distribution of the auriculotemporal nerve on the left. A small area on the right cheek was also ob-

served to sweat, although this had never been noted by the patient. Psychic salivation was easily induced, but the syndrome of flushing and sweating did not occur.

Sweating, distributed usually over the upper lip and nares, may be induced in normal subjects by the application to the tongue and chewing of red pepper or ginger. In this patient these foods resulted in sweating on the upper lip but did not induce sweating in the distribution of the auriculotemporal nerve.

Novocaine injection of the superior cervical sympathetic ganglion on the left resulted in a Horner's syndrome and an absence of sweating in response to heat. In the presence of this block, the auriculotemporal syndrome was observed as before, after the usual stimulus.

Intradermal wheals produced with .05 gamma acetylcholine bromide contained in 0.1 cc. isotonic saline solution produced sweating in the involved area on the left face, but none in the corresponding location on the right.

Saliva was collected from the right and left parotid ducts by means of silver cups attached by suction to the parotid gland duct orifices. No

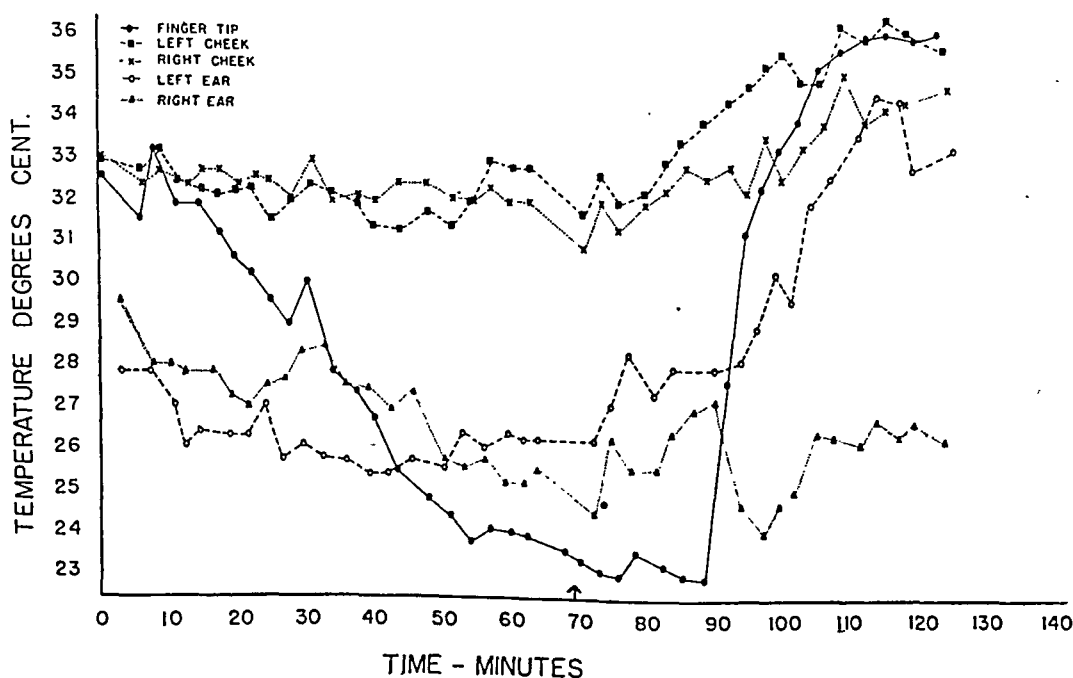


FIG. 2. VARIATIONS IN SKIN TEMPERATURE ON COOLING AND HEATING THE BODY
See text for description of figure.

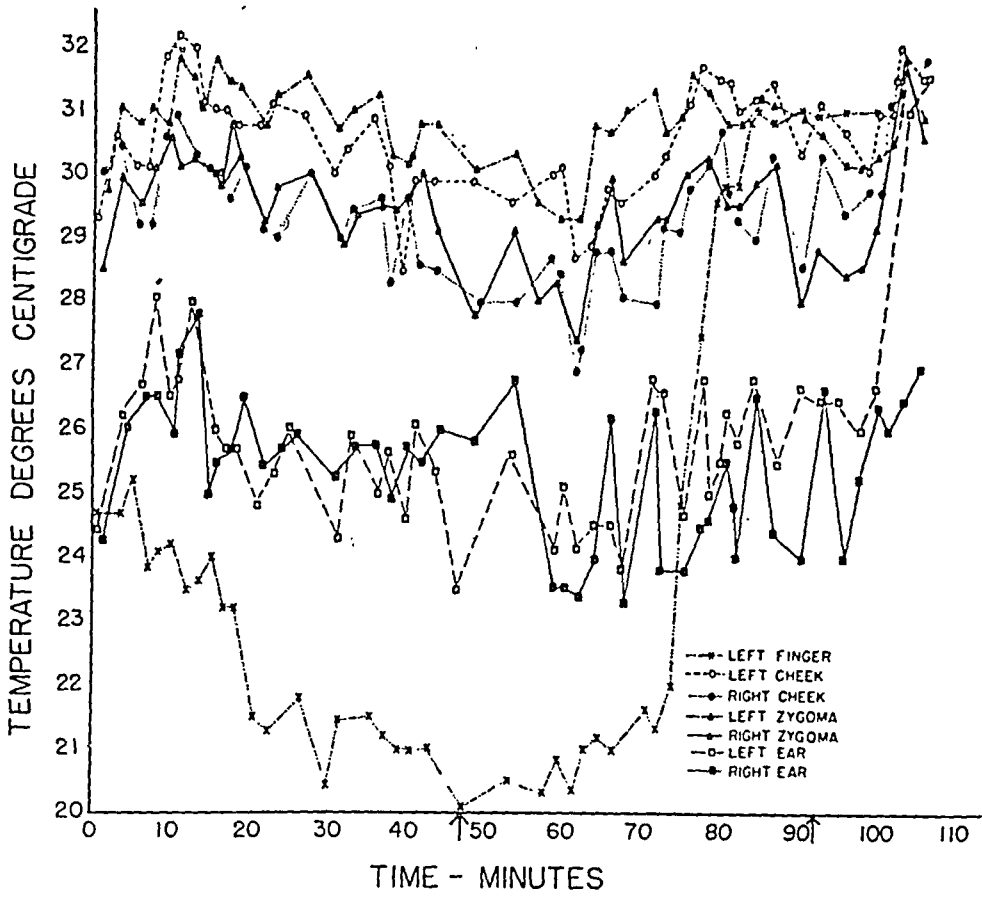


FIG. 3. VARIATION IN SKIN TEMPERATURE ON COOLING AND HEATING THE BODY; VARIATIONS OF THE SKIN TEMPERATURE CONSEQUENT TO EATING IN THE INVOLVED SKIN AREA IN THE AURICULOTEMPORAL SYNDROME
See text for description of figure.

significant differences were found in the two samples in the amount or relative viscosity as measured by high pressure Ostwald viscosimeter.

Studies in the manner described in Case 1 were made of the variations of facial and ear skin temperature (Figure 3) with cooling and warming of the body and extremities. In response to cooling of the extremities and body a distinct fall in finger temperature was obtained. The changes in skin temperature of the ears and face were slight and no significant difference was observed between the right and left sides. At the first arrow (48 minutes) heat was applied and 25 minutes later a distinct rise in finger temperature occurred from approximately 21° C to 31° C. After maximal finger vasodilation in response to heat had been obtained, eating an apple (arrow at 91 minutes) produced a definite increase in temperature not only on the involved left side of the face and ear but also on the right side. No asymmetry of facial sweating in response to heat could be observed.

Observations in Case 3: After the patient chewed an apple and on another occasion a lemon, sweating appeared in the region of the scar 16 and 12 seconds later, respectively. Chewing paraffin induced sweating in the same region in 16 seconds. Chewing ginger resulted in sweating in the region of the scar in 14 seconds and the upper lip in 30 seconds. Intradermal wheals were made in the involved area and in a symmetrical point on the left side of the face. On the right side sweating occurred over and around wheals containing .005 gamma acetylcholine bromide in 0.1 cc. isotonic salt solution. On the left side sweating was absent with wheals of a similar concentration and also with a concentration of 0.05 gamma in 0.1 cc. of isotonic salt solution.

COMMENT

There have been at least 35 instances of the auriculotemporal syndrome reported in the literature (1, 5-15). In the majority of cases the original episode involved both infection in and opera-

tion on the parotid gland. The most common inciting circumstance was a parotitis, complicating typhus or typhoid fever, which had been drained surgically. The syndrome has also followed parotitis complicating syringomyelia, mumps, operation on the parotid without infection (2, 16), and parotitis of unknown etiology in the absence of suppuration and operation. The sweating makes its appearance from a few days (14) to three years (9) after the original episode, and lasts the rest of the patient's life. One case is reported with gradual disappearance of the syndrome over a three-year period (11). Uniformly the sweating appears over part or all of the distribution of the auriculotemporal nerve on the injured side. It is usually accompanied by visible flushing, and patients without visible flushing describe a sensation of warmth over the involved area. Minor disturbances in sensation and decreased sweating response to heat over the distribution of the auriculotemporal nerve of the involved side are frequently noted.

The severity of sweating varies from patient to patient, being slight in some (Case 3) and profuse in others (Cases 1 and 2). The studies described above suggest that relief may be obtained in instances of severe flushing and sweating by interruption of the efferent arc by alcohol injection or surgical section of the auriculotemporal nerve.

Mechanism of auriculotemporal syndrome— anatomic pathway

It appears that the disorder in the auriculotemporal syndrome lies in the efferent arc. The pathway of the afferent arc is variable since sweating may be initiated by stimulation of the seventh or ninth nerves and has been reported to result from psychic stimulation. The efferent pathway involves fibers in the region of the auriculotemporal nerve as demonstrated by the effect of procainization (Case 1). Since procainization of the superior cervical ganglion resulted in the disappearance of sweating in response to heat over the face but did not affect the occurrence of the auriculotemporal syndrome, it is clear that the fibers in the efferent arc do not pass through the cervical sympathetics.

Proposed theories:

I. It has been suggested (17) that the sweating phenomenon results from the diffusion of acetylcholine or cholinergic substances from the injured parotid gland. The speed of the reaction, the distribution in the course of the entire auriculotemporal nerve and the occurrence of the phenomenon in patients in whom the involved parotid gland is in all likelihood completely destroyed (Case 1) are against this interpretation.

II. Needles (12) noted the appearance of the auriculotemporal syndrome coincident with the closure of a salivary fistula, and felt that the syndrome might be caused by the irritation of sudomotor nerve fibers passing through or near the parotid gland by the swelling of the gland on mastication. This appears unlikely, since the syndrome may appear in the presence of an atrophied and functionless parotid gland (Case 1).

III. Ford has suggested (8) that the auriculotemporal syndrome may be explained by injury to the auriculotemporal nerve and subsequent aberrant regeneration of parotid secretory fibers along sudomotor and vasomotor pathways. The auriculotemporal nerve contains, in addition to sensory fibers, secretory fibers to the parotid gland, sudomotor fibers, and vasodilator fibers to the area of sensory distribution of the nerve (18); the parotid secretory fibers, sudomotor and vasodilator fibers are all cholinergic. The following facts are in support of this hypothesis:

1. The time interval between the original injury and the appearance of the syndrome, usually one month or longer, is consistent with a period of nerve degeneration and subsequent regeneration. One case, however, is reported (14) where the syndrome appeared two to three days following injury.
2. The frequently noted abnormalities in sensation and decreased sweating response to heat over the distribution of the auriculotemporal nerve, and, as in one of our cases, defective vasomotor response over the distribution of the auriculotemporal nerve, are consistent with injury to all components of the auriculotemporal nerve. Although hypersensitivity of the sweat glands over the affected region is consistent with the interpretation that the involved sweat glands have been partially denervated, it is difficult to explain the

evident hypersensitivity after regeneration has taken place.

The resemblance of the case described by Uprus, Gaylor and Carmichael (16) to those exhibiting the auriculotemporal syndrome is so striking as to suggest a common mechanism. They described an instance of flushing and gustatory sweating over the distribution of the right cutaneous colli nerve appearing one to two years following the removal of cervical lymph glands through a horizontal incision over the mid right sternocleidomastoid muscle. In the involved area, sensations to pin prick and light touch were diminished. There was diminished sweating response to heat and an impaired vasomotor response after thermal stimuli. Procainization of the cutaneous colli nerve (sensory and vasomotor to the area) had no effect upon the flushing and the sweating reaction induced by food. Blocking the lingual nerve proximal to the origin of the chorda tympani resulted in cessation of salivation and the disappearance of the phenomena. In this instance the primary injury was to the cutaneous colli nerve and injury to the secretory fibres in the lingual nerve appears unlikely because of the level of the incision. With absence of injury to submaxillary secretory fibers, aberrant regeneration is unlikely in this case.

IV. The explanation offered here is that the auriculotemporal syndrome may represent the response of denervated and hypersensitive sweat glands to previously unapparent cranial sudomotor impulses. List and Peet observed (18) marked facial gustatory sweating following postganglionic cervical sympathectomy. In these patients, the sweating appeared following the eating of foods which were similar to those foods causing sweating in patients with the auriculotemporal syndrome. List and Peet (18) suggested that this type of sweating was an "exaggeration of normal gustatory sweating" caused by denervation hypersensitivity of the sweat glands, but they felt that denervation hypersensitivity was not the explanation of the auriculotemporal syndrome, because the diminution of heat sweating over the area involved in the auriculotemporal syndrome was so slight as to suggest that the denervation of the sweat glands was negligible. However, in the

reported cases of the auriculotemporal syndrome, hypersensitivity to pilocarpine as manifested by early sweating in the involved area, and our observations of local hypersensitivity to acetylcholine in the involved regions as demonstrated by intracutaneous wheals or iontophoresis of acetylcholine, clearly show that there is a hypersensitivity of the sweat glands in the involved region. That hypersensitivity to cholinergic substances may occur in organs following denervation has been demonstrated in the submaxillary gland (19), skeletal muscle (20) and iris (21). In these latter studies hypersensitivity developed in from one to two weeks. Observations on the time relationship of the development of hypersensitivity following denervation of sweat glands are necessary.

Other data are available consistent with the interpretation that a cranial supply of sudomotor fibers exists. It is a common observation that facial sweating occurs in normal individuals with the eating of hot spicy foods. More unusually, it may occur with the eating of chocolate (9) and the drinking of vinegar (22). Gustatory sweating in normal individuals, as produced by the eating of ginger, has been studied by us. The sweating occurs maximally about the mouth and nose. Observations were also made in a patient who had a unilateral preganglionic cervical sympathectomy for Raynaud's syndrome. In this patient who had no Horner syndrome, there was absence of heat sweating on the denervated side. Gustatory sweating induced by eating ginger was marked on the normal side of the face but scarcely perceptible on the denervated side. This would suggest that the nervous pathways involved in gustatory sweating consequent upon the eating of ginger passed through the cervical sympathetics. The pathways concerned in the auriculotemporal syndrome do not, as demonstrated by the fact that the syndrome is not affected by cervical sympathetic block. Furthermore in Case 2 sweating induced by ginger was observed to occur independently from the auriculotemporal syndrome sweating. The failure to block the syndrome by procainization of the superior cervical ganglion (Cases 1 and 2), although heat sweating was abolished, is also consistent with the presence of cranial sudomotor fibers. That the facial sweat glands may have a

dual innervation from the brain and cervical sympathetics was suggested by Takino (23) who observed both medullated and non-medullated nerves around the sweat glands of the face. The latter evidence is not decisive, and further studies on this point are necessary.

It would seem, therefore, that the auriculotemporal syndrome, post-sympathectomy gustatory sweating and the case of Uprus, Gaylor and Carmichael represent sweating responses to nervous impulses in sweat glands made hypersensitive by denervation. Although as noted above, in the discussion of Ford's hypothesis, these nervous impulses may be from abnormally regenerated salivary secretory fibers, if cranial sudomotor fibers exist, they might be the efferent pathway in the three types of gustatory sweating under consideration. The three syndromes would then represent complete or partial severance of the sudomotor and vasomotor sympathetics to the involved regions leading to hypersensitivity of the sweat glands and exaggeration of normal and hitherto not obvious cranial sudomotor and vasomotor impulses.

SUMMARY AND CONCLUSIONS

1. The auriculotemporal syndrome is characterized by gustatory sweating and flushing over the cutaneous distribution of the auriculotemporal nerve. Three patients have been studied who exhibited this syndrome.

2. The gustatory sweating and flushing seen in the auriculotemporal syndrome is a manifestation of a reflex in which the efferent arc is through the auriculotemporal nerve or through adjacent autonomic fibers and not through the cervical sympathetic nerves. The afferent arc is non-specific; the syndrome may be induced by stimulating the seventh and ninth nerves.

3. There may be a deficiency in sudomotor, thermo-regulatory and sensory innervation over the involved area.

4. A local hypersensitivity of the sweat glands in the involved region to acetylcholine bromide is present.

5. The mechanism of this syndrome is apparently related to denervation hypersensitivity of the sweat glands analogous to the hypersensitivity

to adrenalin which occurs after post-ganglionic division of sympathetic fibers.

6. In instances of severe flushing and sweating, the described studies suggest that relief may be obtained by interruption of the efferent arc by alcohol injection or surgical section of the auriculotemporal nerve.

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STUDIES IN METHIONINE AND SULFUR METABOLISM. I. THE FATE OF INTRAVENOUSLY ADMINISTERED METHIONINE, IN NORMAL INDIVIDUALS AND IN PATIENTS WITH LIVER DAMAGE^{1,2}

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The cardinal role of the liver in protein and amino acid metabolism has been well established. Consequently, studies of amino acid metabolism occupy a natural and fundamental place in an investigation of metabolic aberrations in hepatic disease. In 1913, Van Slyke and Meyer (1) demonstrated that certain amino acids when injected intravenously are rapidly removed from the circulation in the normal animal. They also demonstrated that a considerable capacity for temporary storage of amino acids existed in certain tissues, notably liver and muscle. Tolerance to various individual amino acids has been studied in connection with hepatic function. Thus Jastrowitz (2) in 1908 reported a greater urinary content of glycine in patients with liver damage than in normals following oral ingestion of glycine. A tolerance test to orally administered tyrosine was proposed by Bernhart and Schneider (3) as an aid to the diagnosis of hepatic disease. Similar procedures with single and multiple amino acid preparations have been studied by other investigators (4, 5). The recent development of specific and sensitive microbiologic procedures for the assay of amino acids (6) makes possible the further study of this problem.

By virtue of its prominent role in liver physiology, in the work here reported methionine was selected for a study of amino acid metabolism in normal and hepatopathic individuals. The presence of sulfur in the molecule confers an additional

advantage since this serves as a "label" in the interpretation of the metabolic fate of the administered methionine.

In the present study a standard dose of *DL*-methionine was administered intravenously. Observations were then made on the rate of disappearance of the amino acid from the plasma together with a comparison of the excretion of free methionine and sulfate sulfur before and after the substance was given. Since the racemic *DL*-methionine was used, it was of interest to follow the metabolism of both isomers. This was made possible by the separate analysis of samples by two micro-organisms, one utilizing only the *L*-isomer, the other, both *D*- and *L*-isomers.

METHODS

All patients (veteran and naval personnel) received a standard high protein, high carbohydrate intake over a two-day period preceding the test, during which time all medication was discontinued. They received no food from 8:00 p.m. the preceding night until after the 180-minute blood and urine samples had been obtained. Methionine determinations were made on deproteinized filtrates of plasma obtained from heparinized blood. After removal of a blood sample for determinations of the fasting methionine level, 50 cc. of a 3 per cent *DL*-methionine solution were injected over a five-minute period. Blood samples were then drawn at 15, 30, 60, 120, and 180 minutes after the injection. Urine samples corresponding to a two-hour period prior to the administration of the test dose of methionine as well as to the subsequent three-hour period were collected and assayed for methionine and, in some cases, sulfate sulfur (both free and total), and total amino nitrogen. This latter constituent will be discussed in a later communication. In several instances, methionine excretion during various periods up to 48 hours after the test dose had been given was measured.

Methionine was determined microbiologically using as test organisms *Leuconostoc mesenteroides*, P-60, for the assay of *L*-methionine and *Lactobacillus fermenti*, 36, for

¹ This work has been performed under a contract between the Office of Naval Research and the University of California.

² The biotin, pyridoxal, and pyridoxamine used in this study have been supplied through the courtesy of Merck & Co., Inc.; the methionine, through the courtesy of Wyeth, Incorporated.

assay of *DL*-methionine. Apparent *D*-methionine concentrations were obtained by difference. All other analytical details were as described in previous publications from this laboratory (7, 8). Standard chemical methods were used for the sulfur and amino acid determinations (9, 10).

RESULTS

The data here presented represent observations over a 15-month period on normal men and in men with acute, chronic, convalescent, and healed (inactive) liver disease.

Fasting methionine values

The plasma fasting *L*-methionine levels varied over a considerable range, from 0.22 to 1.48 mg. per cent in normal controls with an average of 0.73 ± 0.07^a mg./100 cc. The average for pa-

tients, if values at all stages of the disease are included, is higher than that of normals. Certain patients with very severe, active liver disease had extremely high fasting values, up to 4.00 mg. per cent, and an occasional patient who had received oral methionine in the recent past, had still higher plasma *L*-methionine levels; but over 90 per cent of the fasting values in patients fell in the control range. Except for those patients with very high values, no particular significance can be attached to the fasting levels, since a high degree of variability in fasting blood methionine is apparently the usual situation. Contrary to expectation, some individuals had measurable fasting levels of *D*-methionine.

Observations on normal subjects

Figure 1 illustrates the variations in plasma *L*- and *D*-methionine as observed at various intervals

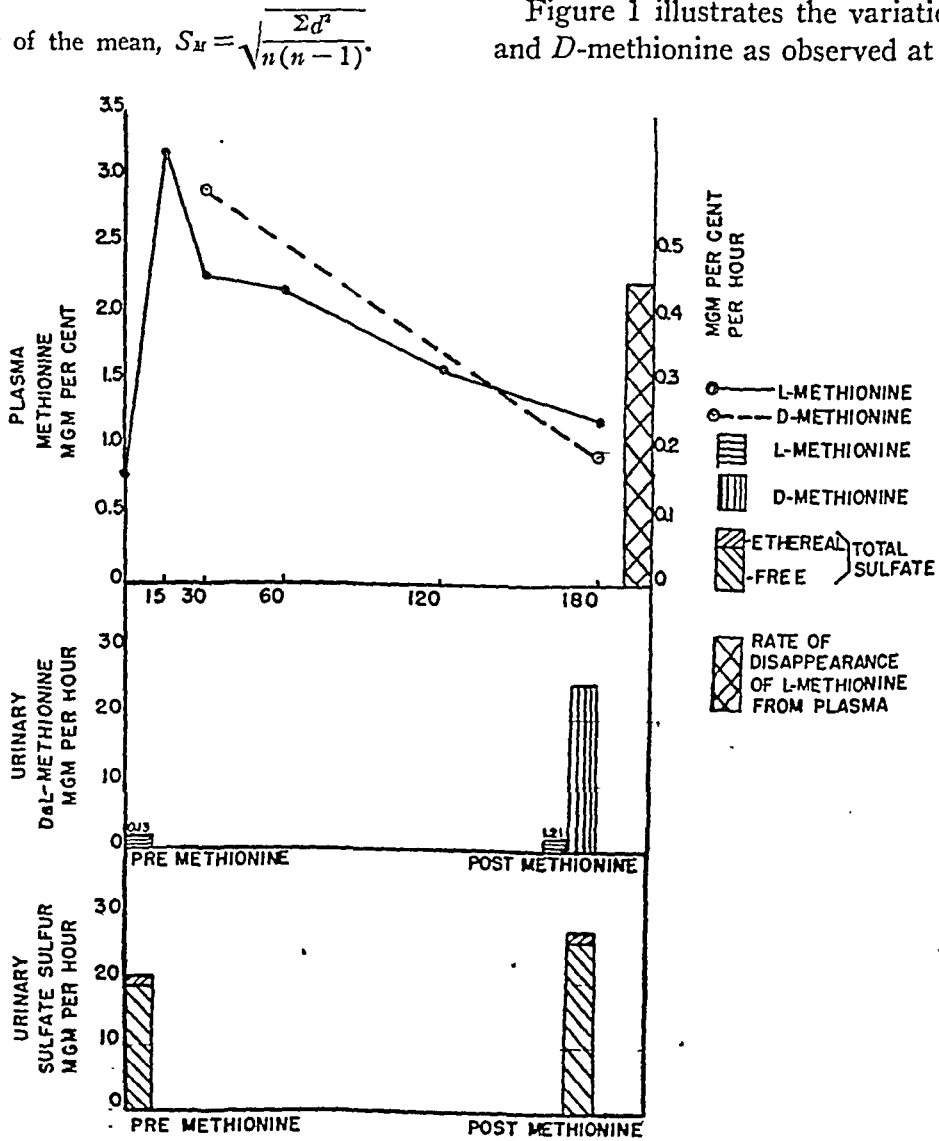


FIG. 1. PLASMA AND URINARY *D*- AND *L*-METHIONINE, AND URINARY SULFATE, PRIOR AND SUBSEQUENT TO METHIONINE INFUSION IN NORMAL INDIVIDUALS

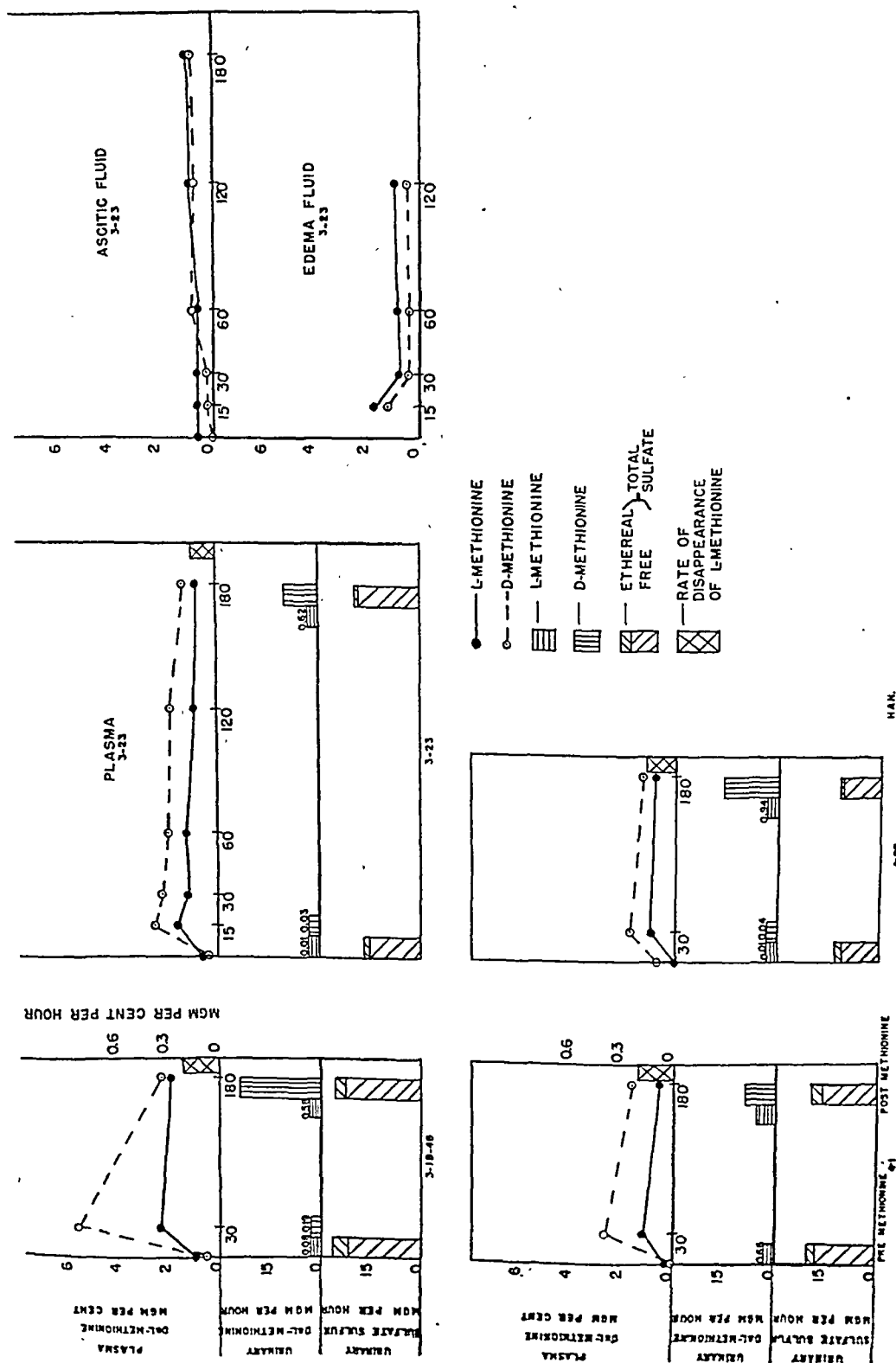


FIG. 2. SERIAL PLASMA AND URINARY METHIONINE, AND URINARY SULFATE DETERMINATIONS IN A CIRRHOTIC, INCLUDING (ON 3/23/48) SIMULTANEOUS ASCITIC AND EDEMA FLUID METHIONINE DETERMINATIONS

It is to be noted that the edema fluid levels parallel those of the plasma.

after the intravenous infusion of the methionine solution in normal subjects. It will be noted that the peak values obtained 15 minutes after the injection, decline rapidly up to the 30-minute post-injection period, then more slowly and regularly over the succeeding 150 minutes. Apparently there is a very rapid initial diffusion of the amino acid into extravascular spaces, since even a very short time after the test dose was given, less than one-fourth of the injected material could be accounted for in the plasma. The rapid disappearance of *L*-methionine from the plasma during the 15 to 30-minute period is presumably referable to diffusion plus utilization. The slow, steady drop in plasma *L*-methionine concentration over the 30 to 180-minute period is attributed to utilization alone (anabolism plus catabolism). This interpretation is buttressed by observations on interstitial fluid methionine levels in a patient with cirrhosis (Figure 2). The actual figures obtained for the various points on the curve (in Figure 1) varied in different individuals since a fixed test dose was used regardless of variation in body size. However, by expressing the rate of disappearance as mg. of plasma methionine on a per hour basis, calculated for the 2½-hour period between the 30- and 180-minute points, it was possible to reduce all studies to a comparable equivalent. In an original series of 11 normal individuals the average rate of disappearance was thus calculated to be 0.41 ± 0.03 mg. per cent per hour for the *L*-isomer (7). The subsequent study of several other normals when added to this original series yielded a rate of 0.45 ± 0.03 mg. per cent per hour. This latter figure has been used as an average normal standard in the comparative studies of methionine metabolism in liver disease to be discussed in the present paper.

Figure 1 also details the average hourly excretion of *DL*- and *L*-methionine as well as sulfate sulfur, total and ethereal, measured for the two-hour period preceding the methionine injection and during the subsequent three hours. Negligible quantities of *L*-methionine were excreted at any time. After injection, a maximum of only 3.36 mg. per hour, with an average of $1.12 \pm .22$ mg. per hour, was found. This indicates that even though the plasma *L*-methionine level was raised

as much as five times the normal fasting level, the maximal rate of renal tubular reabsorption was not reached.

The curve illustrating the average *D*-methionine levels at intervals after injection of the test amino acid indicates that although the peak values are higher, those obtained at 180 minutes tend to be lower than the comparable *L* values. Thus a higher rate of disappearance for the *D*-isomer was usually observed. Inspection of the data on rates and extent of excretion of the *D*-isomer shows that a very considerable quantity is excreted during the initial three hours and this fact probably accounts in large part for the more rapid removal of this isomer from the plasma. However, so far no consistent pattern for the *D*-isomer either in rates of disappearance from the plasma or excretion could be detected. Hence, only the *L* form has been considered in evaluation of metabolic abnormalities in hepatic disease.

Urinary sulfate sulfur excretion during the two hours preceding and the three hours following methionine administration has been utilized as an index of the catabolism of endogenous, and exogenous-plus-endogenous sulfur-containing amino acids, respectively. In an initial group of six normal individuals an average pre-methionine rate of 20.3 mg. per hour was obtained, rising to a rate of 28.0 mg. per hour during the three-hour post-methionine period. Since the sulfur content of the 1500 mg. of methionine administered is 322 mg., it would seem that little of the methionine is catabolized during the initial three-hour period.

Observations in liver disease

It was anticipated that an acutely damaged liver could not as readily utilize administered methionine as the normal organ. The observations to follow support this hypothesis.

(a) *Acute Hepatitis.* Initial values from 14 patients in the pre-convalescent phase of the disease are included in Figure 3. It will be noted that the average rate of disappearance of methionine from the plasma in these patients is slower than that for the normals. Ten of these patients had rates ranging from 0.13 to 0.26 with an average for all 14 patients of 0.26 ± 0.02 mg. per cent. When compared to the average normal figure of 0.45 ± 0.03

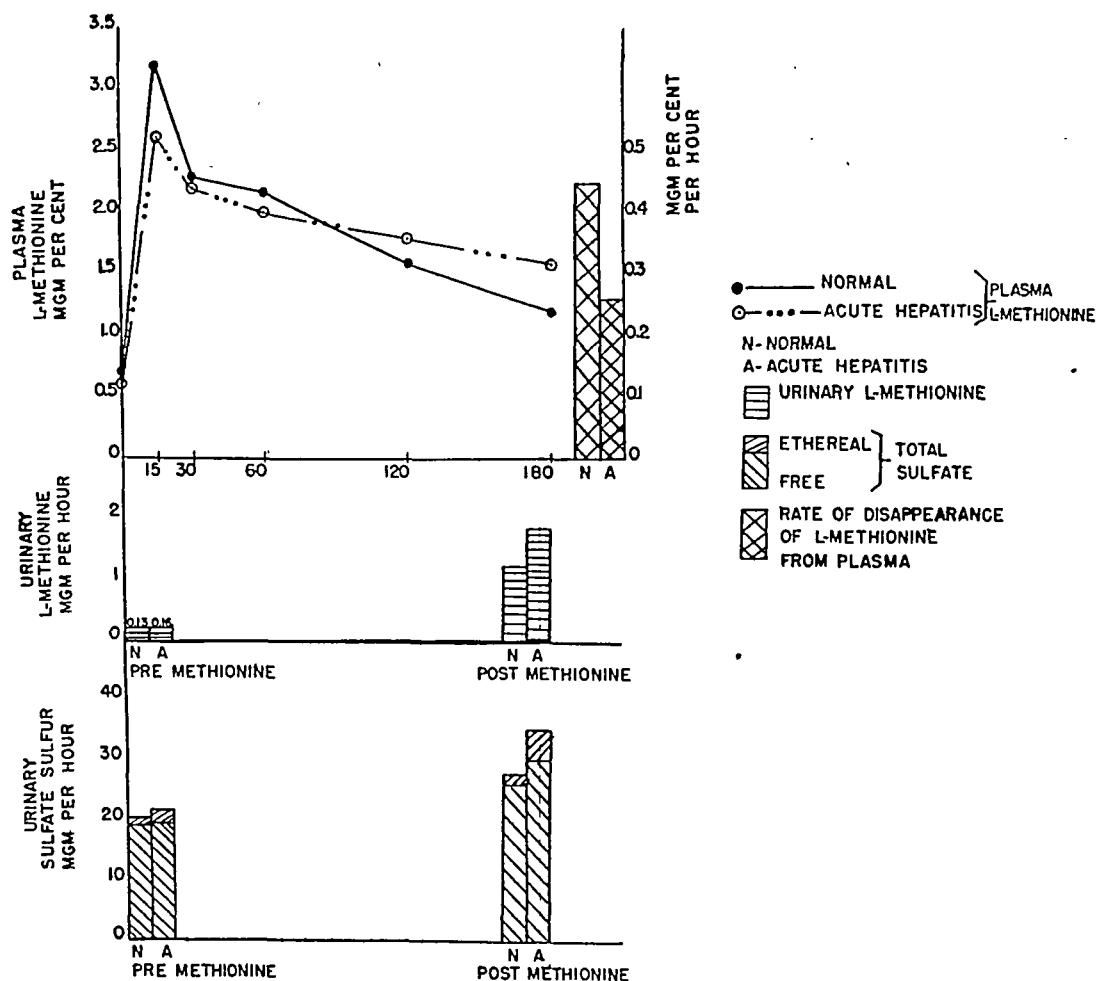


FIG. 3. COMPARISON OF *L*-METHIONINE AND SULFATE VALUES IN NORMAL INDIVIDUALS, AND IN PATIENTS WITH ACUTE HEPATITIS

mg. per cent, this difference was shown to be statistically significant ($D_M:S_{D_M} = 4.7$).⁴

The urinary excretion of *L*-methionine after the test dose averages $1.83 \pm .52$ mg. per hour as compared to $1.12 \pm .22$ in normals. For the two-hour fasting period prior to the test dose, the hepatitis patients had an average excretion of 0.16 mg. per hour as compared to 0.12 in normals. Neither of these differences has statistical significance.

⁴This refers to the ratio of the difference of the means (D_M) to the standard error of this difference (S_{D_M}), calculated as follows: $S_{D_M} \sqrt{\frac{\sum d_1^2 + \sum d_2^2/n_1n_2}{(n_1 - 1)(n_2 - 1)/n_1 + n_2}}$, where $\sum d^2$ represents the sum of the squares of the differences from the mean and " n " equals the number of cases. When this ratio exceeds 3, results are considered significant.

Inorganic sulfate sulfur excretion in four of the acute hepatitis patients averaged 22.0 mg. per hour pre-methionine and 35.4 mg. per hour post-methionine. Insufficient data are at hand to permit of valid statistical evaluation at this time.

(b) *Convalescent Hepatitis*. As convalescence progresses in the patient with acute hepatitis, the rate of disappearance of administered *L*-methionine rises to normal or super-normal levels and the urinary sulfate excretion approaches the normal pattern. These findings are included in serial observations in a representative patient during the acute and convalescent phases of a moderately severe hepatitis (Figure 4). The clinical course of the disease is indicated by the total serum bilirubin levels. During the early acute phase of the disease, it will be noted that in addition to a minimal rate

of disappearance of plasma *L*-methionine, the fasting *L*-methionine levels were markedly elevated—to more than four times the average fasting level. Return to a normal fasting level preceded the restoration of a normal rate of disappearance.

(c) *Chronic Hepatitis*. The diagnosis of chronic viral hepatitis is arbitrarily assigned to those patients who, over a period of six months or longer have failed to recover from an attack of established or presumptive hepatitis. In view of the obscure etiology of the syndrome and the likelihood of progressive hepatocellular damage and replacement fibrosis (cirrhosis) in such individuals, these patients represent a small but important segment of the entire group. In Figure 5, data on three such individuals are presented. The rate of disappearance of plasma methionine is normal in all

but one instance. A high fasting methionine is noted on one occasion in the man with the most severe hepatitis together with a very high level of urinary sulfate, then and subsequently. The data in these patients is still inadequate for any but inferential purposes. A high rate of catabolism may explain the “normal” rates (*vide infra*).

(d) *Chronic Liver Damage (Cirrhosis)*. The term “cirrhosis” is used in referring to patients under study in this group. All patients so designated have extensive hepatic fibrosis with or without fatty infiltration, with varying degrees of active hepatocellular damage. The exciting etiology in the majority of instances is chronic alcoholism with resultant and/or concomitant dietary insufficiency, continuing or recurring over a period of many years. Some of the patients have had

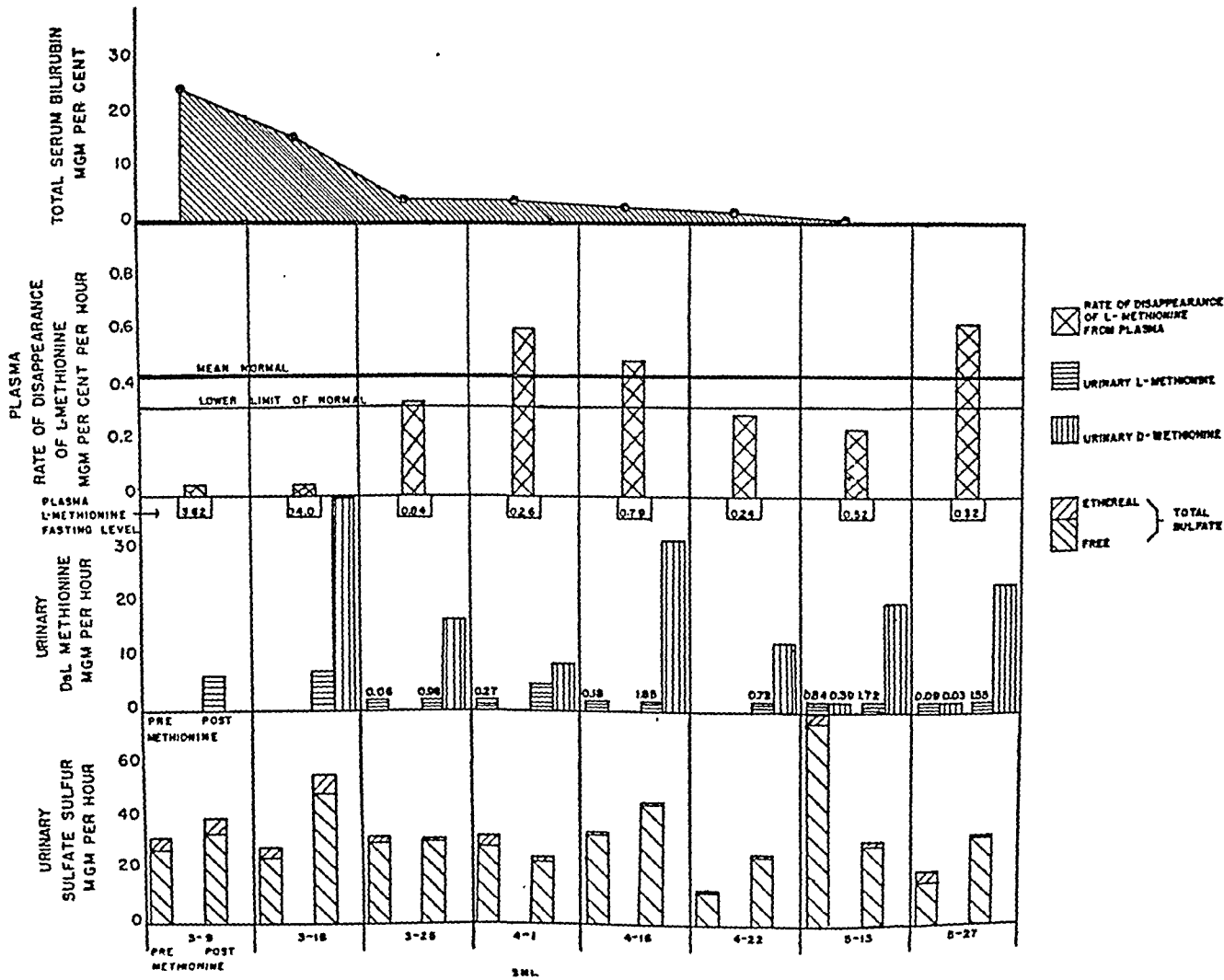


FIG. 4. SERIAL EVALUATION OF METHIONINE-SULFUR METABOLISM DURING THE COURSE OF AN ATTACK OF ACUTE HEPATITIS IN PATIENT SMI

The clinical status of the disease is indicated by the serum bilirubin levels.

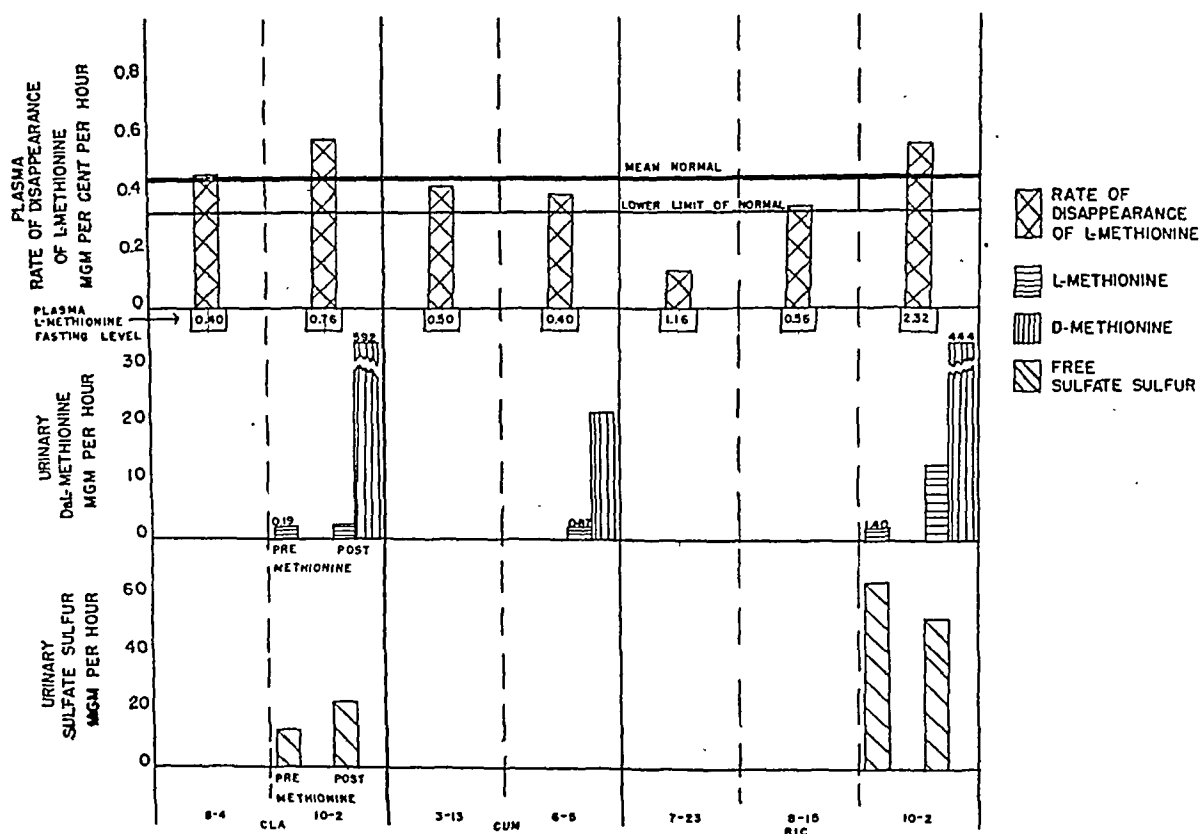


FIG. 5. CHRONIC VIRAL HEPATITIS; METHIONINE AND SULFATE STUDIES IN THREE PATIENTS

marked degrees of ascites, with or without other manifestations of portal hypertension. The diagnosis has been proven in all instances by one or more biopsies. We regard these patients as being most satisfactory for controlled metabolic studies because of "known" etiology, absence of unpredictable fluctuation in clinical and biochemical status, and because of the existence of a "panel" of tests which roughly but adequately reflect the histological and physiological status of the liver.

The same procedures and technics were used as in the preceding groups of patients. Figure 6 shows the average values initially obtained in a group of ten such patients, in three of whom only fasting levels were obtained.

Considering only these determinations, an average rate of disappearance of 0.26 ± 0.04 was obtained. The difference between this initial rate and that already noted in normals is statistically significant ($D_M:S_{DM} = 3.2$). When 26 values obtained in 12 patients at all stages of the disease were averaged, a mean rate of 0.22 ± 0.05 mg. per.

100 cc. per hour resulted. Statistical comparison of this figure with that obtained in normals reveals a ratio of D_M to S_{DM} of 10—a highly significant difference. The inclusion of only the initial values in construction of Figure 6 is done for the sake of uniformity; i.e., to permit of proper comparison between these data and those obtained in hepatitis patients.

L-methionine excretion averaged 0.163 mg. per hour before injection, and 0.770 mg. per hour post-injection, figures not significantly different from the control values. This statement also applies to values obtained in all stages of the disease.

The urinary sulfate values included in Figure 6 are too few for significance. In Figure 7 are shown a series of pre- and post-methionine urinary sulfate values obtained in a group of patients with chronic liver damage. It will be noted that the majority of these values fall within the normal range. Two highly abnormal values are included at this time only for purposes of brief comment (RIC and DRE). While both had high

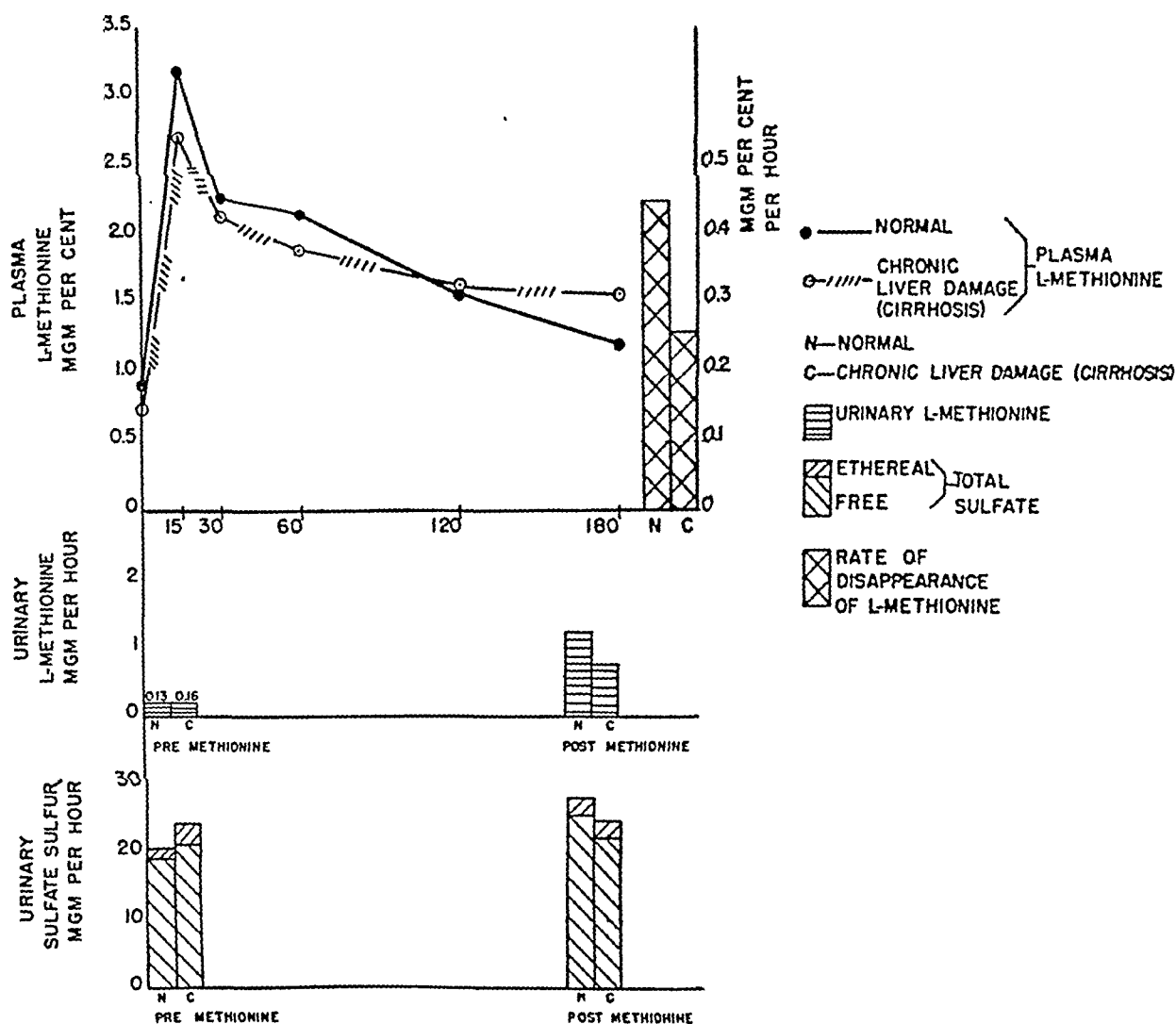


FIG. 6. COMPARISON OF L-METHIONINE AND SULFATE VALUES IN NORMAL INDIVIDUALS, AND IN PATIENTS WITH CHRONIC LIVER DAMAGE (CIRRHOSIS)

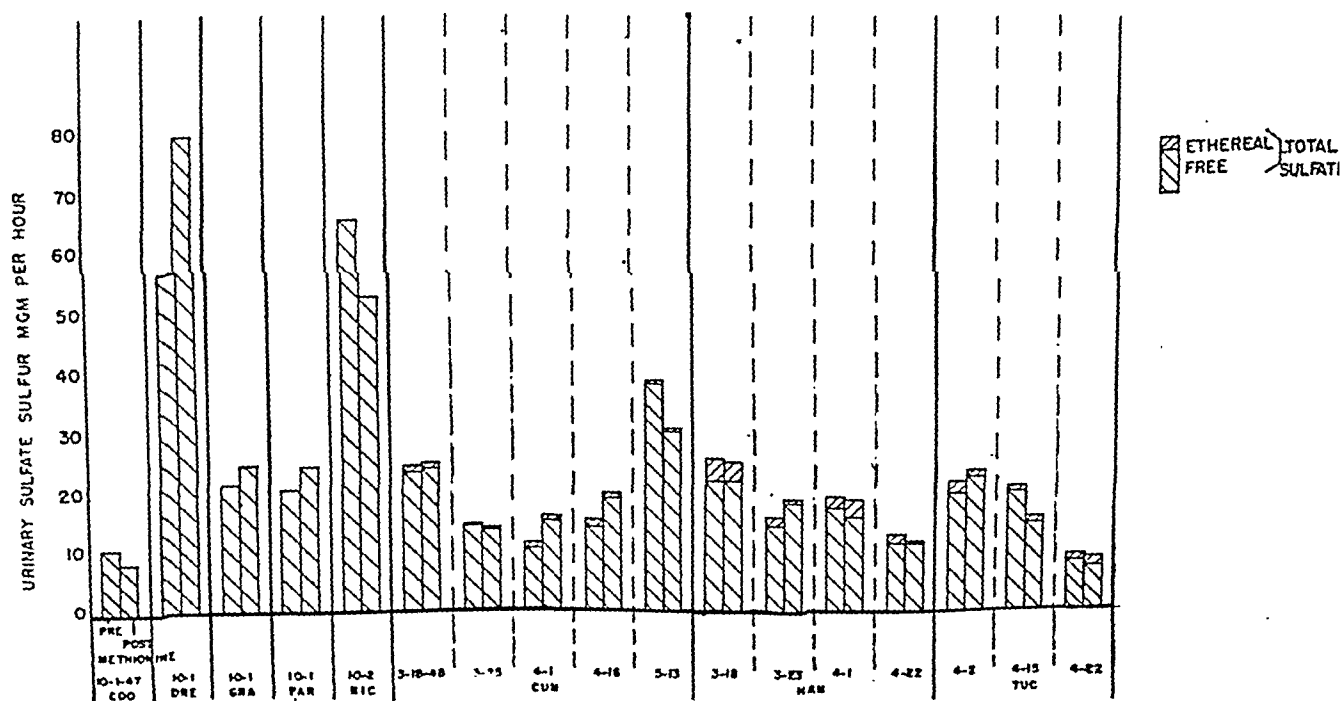
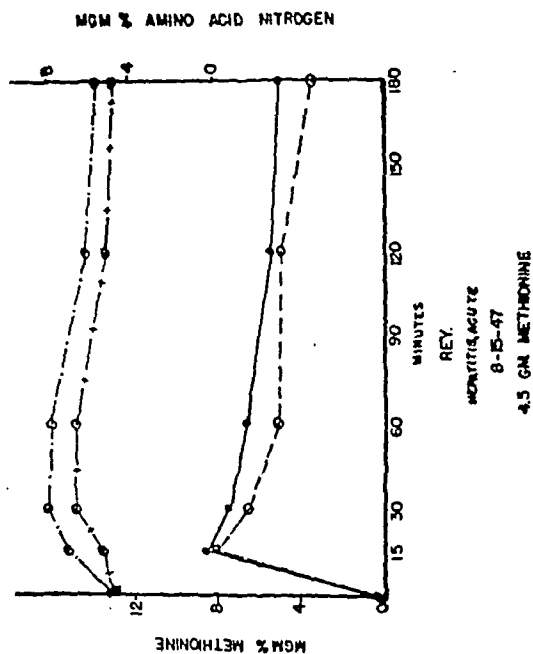


FIG. 7. URINARY SULFATE, PRE- AND POST-METHIONINE, IN A GROUP OF PATIENTS WITH CHRONIC LIVER DAMAGE

THE FATE OF INTRAVENOUSLY ADMINISTERED METHIONINE



PLASMA VALUES FOR α AND L METHIONINE
AND TOTAL AMINO NITROGEN

AFTER I.V. INJECTION OF VARYING DOSES OF α METHIONINE

Amino Acid N
Amino Acid N Corrected For
Plasma α -Methionine - · - · -
 α -Methionine -----
L-Methionine - - - - -

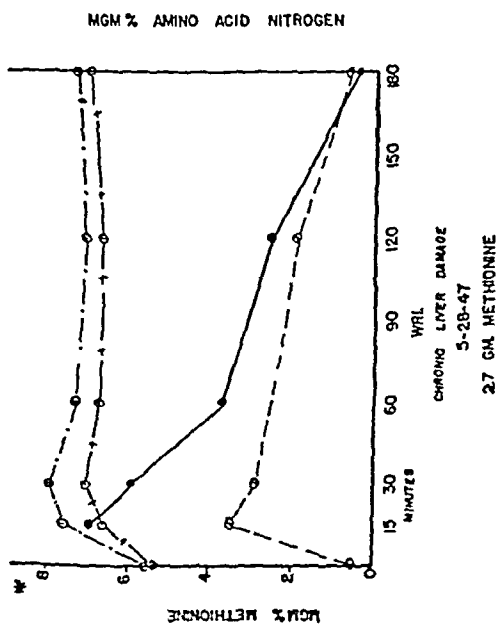
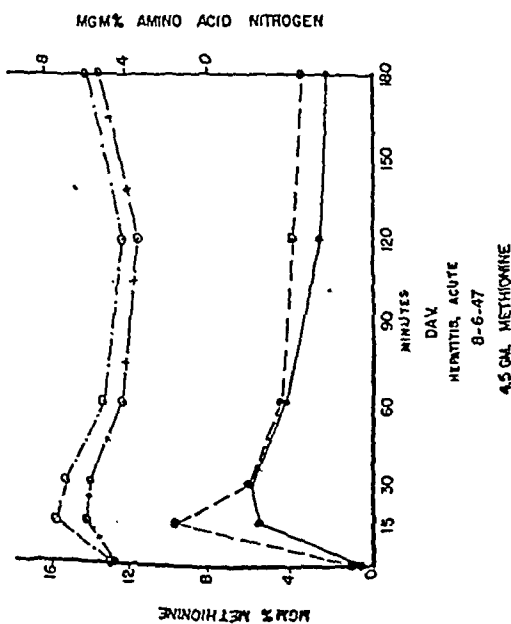


FIG. 8a, b. PLASMA AND URINARY METHIONINE LEVELS IN RESPONSE TO INFUSION OF VARYING AMOUNTS OF METHIONINE

See next page for Figure 8b

pre-methionine values, there was a sharp fall in the post-methionine sulfate in RIC (chronic hepatitis) and a sharp rise in DRE. Patient RIC has made some response to therapy. DRE died of acute hepatic insufficiency about six weeks later.

The use of larger doses of methionine

The original selection of 1.5 grams of *DL*-methionine as the intravenous dose was highly arbitrary based upon no more profound reason than that the water solubility of the material at room

temperature was about 3 per cent, and that a 50-cc. syringe was the largest syringe with which one could conveniently work. That this dose proved to be an amount which answered many of our questions, has been a source of gratification.

A few patients have received larger dosages—up to 4.5 grams (150 cc. of a 3 per cent solution) given under the same conditions as those previously described for the smaller amount. The data so obtained indicate that the initial 15-minute values for *D*- and *L*-methionine are roughly proportional to the dose; that the disappearance from the plasma of *L*-methionine follows the same pattern as in the case of the smaller amount; that there is not a significant increase in urinary *L*-methionine, but that the urinary *D*-methionine content is roughly proportional to the amount administered (Figures 8a and 8b). The net result of this small number of observations was to assure us that a larger, less convenient dose had no physiological advantage.

DISCUSSION

The purpose of this paper is to present certain fundamental observations on methionine metabolism, particularly insofar as this subject is related to normal and abnormal liver physiology in the human subject. The possible clinical implications of these data will be considered in a separate communication, in which correlation of these procedures and of certain standard tests of liver function will be stressed.

Urinary excretion

One would expect that the natural isomer of an essential amino acid would have a high priority rating in terms of renal tubular reabsorption. Such is the case in man with *L*-methionine as adequately demonstrated in all subjects studied and with all doses used. Furthermore, that the renal physiology of *L*-methionine is not significantly altered by acute or chronic liver damage is likewise well demonstrated, despite the major differences in the rate of "utilization" of the *L*-isomer, as evidenced by blood studies, in many patients with liver damage. Reports on urinary methionine content (11, 12), therefore probably have no significance in terms of normal or abnormal liver physiology.

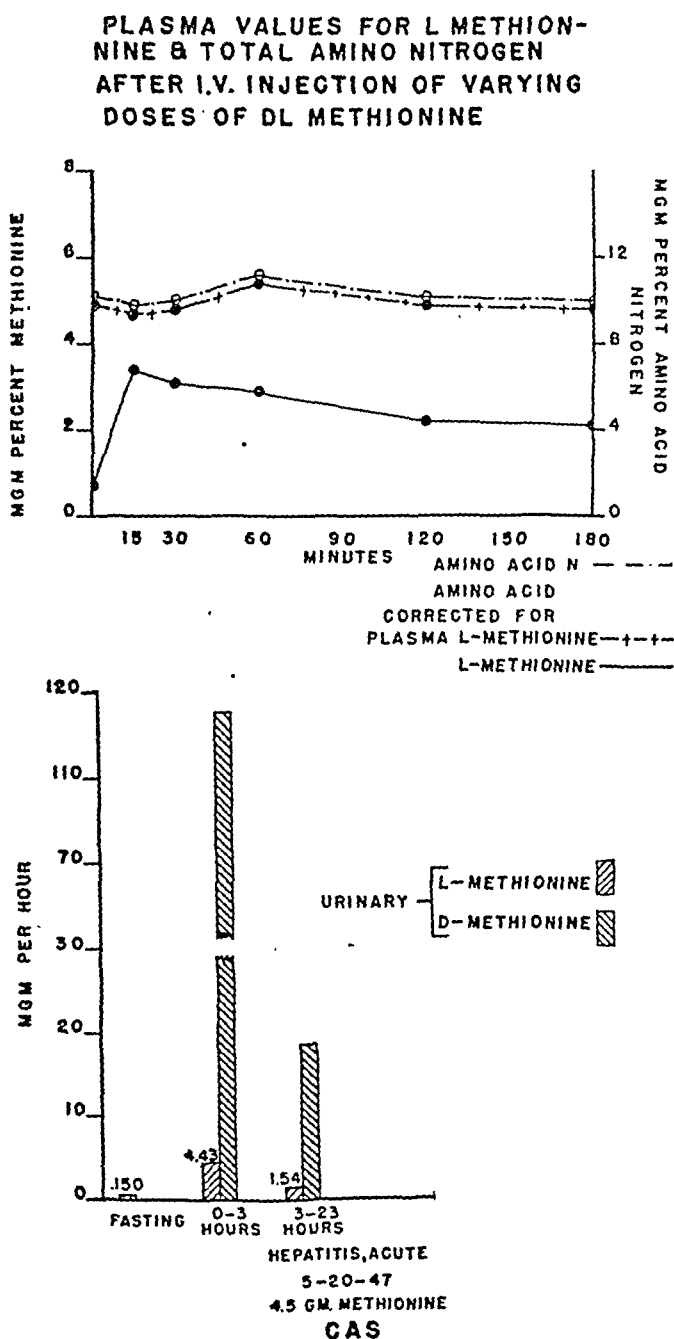


FIG. 8b

See legend, Fig. 8a

The fate of D-methionine

The interpretation of the data obtained on plasma and urinary *D*-methionine is difficult. It is apparent that rapid and considerable renal excretion occurs in all instances. Such data as are available on urinary methionine in patients who received the 4.5 gram dose of *DL*-methionine, suggest that a mathematical relationship does exist between the size of the dose of the *D*-isomer and the urinary content of this material, as contrasted to the natural isomer. Whether biological tubular reabsorption exists is still to be determined. In any event, it appears that the rate of disappearance from the plasma, while, on the average, more rapid than that of the natural isomer, cannot be accounted for on the basis of renal excretion alone, nor can it be accounted for on the basis of total catabolism of the methionine molecule. Hence, anabolism of *D*-methionine must occur. Whether such metabolism initially requires deamination followed by transamination to the *L*-isomer is unknown to us at the present time. The presence of a specific enzyme (*D*-amino acid oxidase) in liver and kidney which initiates such a reaction is well known. Quantitation of keto acid production and of other metabolites, now in progress, may help to throw further light on this problem.

Fasting plasma methionine

An additional factor which deserves emphasis in regard to *L*-methionine is the high fasting level noted in a few patients with acute hepatitis and during the acute phase of chronic liver damage, e.g., Patient SMI (Figure 3). As the disease subsides, the elevated level returns to normal well before a normal rate of utilization is achieved. The analogy to the behavior of blood dextrose in diabetes is obvious. Work now in progress indicates that very high fasting plasma *L*-methionine levels in cirrhotics may be found for several days after the discontinuance of oral methionine.

L-methionine metabolism

The evidence in support of the interpretation of the rate of fall during the 30 to 180-minute period is also worthy of further discussion, as such an interpretation is still to be proved. That the rapid fall during the 15 to 30-minute period is largely referable to diffusion, is well evidenced by

the plasma-interstitial (edema) fluid v. tained in patient HAN (Figure 2). It is also parent from the same data that once this initial diffusion has occurred, equilibrium is maintained between these two phases of body fluid. As already noted no phase of the *L*-methionine plasma curve is referable to urinary excretion and hence only two interpretations of the 30 to 180-minute fall appear to be possible, viz., metabolism or intracellular storage. It would appear unlikely that cellular storage of methionine, as such, would be influenced significantly by impaired liver function and thus only one interpretation remains, viz., utilization (anabolism or catabolism).

Because of the fortuitous presence of sulfur in the methionine molecule, and the mandatory excretion of this sulfur as urinary sulfate when the amino acid is metabolized, one can speak with more assurance of catabolism than of anabolism. From the urinary sulfate data which we have so far obtained, one can say that no significant catabolism of the infused methionine occurs over the three-hour post-infusion period, with a single exception (DRE, Figure 7). That this exception occurred in a man who died of hepatic insufficiency a few weeks later is probably of considerable importance. This man, to our initial discomfort, had a normal disappearance rate. In retrospect, we believe that this finding was referable to two factors—the excessive rate of catabolism, and the presence of a huge amount of ascites at the time the test was done. Since the majority of cirrhotics with ascites, but without evidence of excessive catabolism, have had abnormally slow rates, it is probable that the catabolic factor was of more importance in the production of a “normal” rate of disappearance than was the increased interstitial fluid volume.

“False normal” rates of disappearance of the *L*-isomer have also been noted in a few instances in men who have had abnormally high fasting levels. It may be that one should not attempt to evaluate the utilization rate until the fasting level has returned to normal.

One further word concerning anabolism-catabolism: the small amount of urinary sulfate data which have so far accumulated in normal individuals suggests that little of the administered methionine is catabolized during the first three

pr

hours. This could be construed as an anabolic effect. Nitrogen-sulfate ratio determinations now in progress should support or refute this impression. A number of the acute and chronic liver patients have excreted less urinary sulfate after the methionine than in the basal, pre-methionine period. It is our present feeling that this serves as a significant index of anabolic effect. Confirmation of this impression, and of other of our present interpretations of the data presented, await the accumulation of further pertinent information in the patients here presented, and in others currently under study.

SUMMARY AND CONCLUSIONS

1. Normal human subjects remove intravenously administered methionine from the plasma at a predictable rate.

2. This rate of removal is impaired in many individuals with liver damage.

3. On the basis of the evidence presented, it seems probable that the rate of removal is synonymous with the rate of utilization (anabolism and/or catabolism).

4. Urinary sulfate may serve as an anabolic/catabolic index.

5. Under all conditions studied, the urinary excretion of *L*-methionine is negligible, while that of *D*-methionine is considerable.

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LETTER FROM THE EDITORS

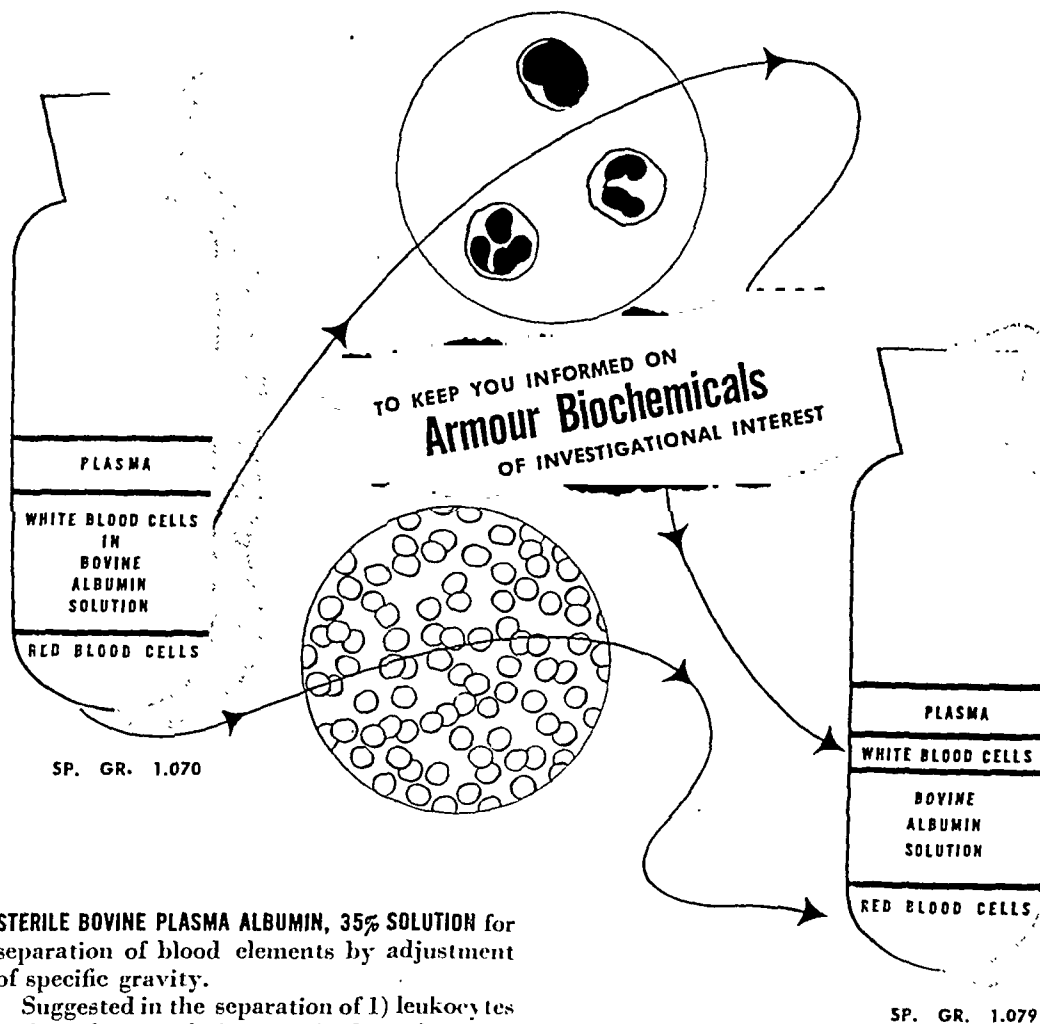
What are the responsibilities of the head of a department with reference to the manuscripts written by his junior colleagues? This question has arisen many times during the deliberations of the Editors over the acceptability of various manuscripts. It is taken for granted that the head of a department is responsible for acquiring the funds for the particular study; for ensuring that the plan and conduct of the investigation is in accord with the best of scientific criteria; for carefully checking the accumulated data and the deductions derived from the data; and for ascertaining that the final conclusions are consistent with the facts noted in the study. Does his responsibility stop at that point? Too frequently that seems to be the case.

On numerous occasions, junior and even senior members of a department have sent us manuscripts which were objectionable because of one, many or all of the following: incorrect spelling, inaccurate grammar, poor idiomatic expression, erasures, corrections in ink or pencil, inconsistent abbreviations, incorrect citation of literature, incorrect references to charts and figures within the manuscript and a variety of other indices of carelessness and untidiness (not excluding dirty fingerprints all over the manuscript). Were these found also in the manuscripts sent us by the head of the department, then one could be critical of the department as a whole. Usually this is not the case, since the manuscript by the "chief" will have none of the above while that of his junior colleagues may be full of them!

We are in complete accord with the principle that it is not our function to dictate the style that an author should employ in the presentation of his studies. However, it is our function to take cognizance of the manner in which the data are presented, the care with which the deductions are derived, the validity of the references, as well as other factors which determine the acceptability of a manuscript. Irrespective of the degree of objectivity with which an editor or reviewer may wish to examine a manuscript, his attitude toward the data and deductions will be affected by the appearance of the manuscript and the manner in which it is written. When confronted with a "sloppy" manuscript he cannot help but wonder whether it reflects an inaccurate worker whose data are the result of careless experimentation.

There is little question but that it is the responsibility of every investigator to prepare his manuscript with the same diligence and care that he put to his investigation. However, many young investigators have not been exposed to the same degree of discipline about the preparation of manuscripts as they have been about the performance of experiments. As J. F. Fulton put it in his discussion of "The Principles of Bibliographic Citation" (Bull. Med. Lib. Assoc., 22, 183, 1933) "formal instruction is seldom given in this basic part of scholarship." It is the responsibility of the head of the department to make certain that the young members of his department are given this essential training. An expenditure of time and money goes into every investigation. The expenditure of just a little more time by the head of the department in the form of critical examination and aid with the preparation of the manuscript, and of a little more money in the form of more adequate secretarial assistance will not only improve the character of the manuscript but will result in a more objective and sympathetic criticism by the unbiased reader.

THE EDITORS



STERILE BOVINE PLASMA ALBUMIN, 35% SOLUTION for separation of blood elements by adjustment of specific gravity.

Suggested in the separation of 1) leukocytes and erythrocytes¹; 2) parasitized erythrocytes and normal erythrocytes²; and 3) eosinophil types³.

BOVINE HEMOGLOBIN ENZYME SUBSTRATE POWDER standardized for proteolytic enzyme assay.

Suggested as a substrate for assay of pepsin¹ and trypsin.

ALPHA MONOPALMITIN, CRYSTALLIZED⁵. Suggested as a substrate for esterase activity and wherever a fatlike compound in a high degree of purity may have application.

¹ Vallec, Hughes, and Gibson: *Blood J. Hemat. Spec. Issue 1*, 82-87 (1947).

² Ferrebee and Geiman: *J. Infectious Dis.* 78, 173-179 (1946).

³ Favour: Private communication.

⁴ Anson and Mirsky: *J. Gen. Physiol.* 16, 59-63 (1932).

⁵ Munson et al. *Fed. Proc.* 6, 230-281 (1947).

ALSO: Crystallized Enzymes (pepsin, trypsin, chymotrypsin, ribonuclease, lysozyme), Bilirubin (crystallized and amorphous), Adenosine Triphosphate (muscle), Fraction I (bovine fibrinogen), and other fractions from bovine and porcine plasmas, Hemin Chloride, and Catalase, Technical Grade.

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MEASUREMENT OF GLOMERULAR FILTRATION RATE IN PREMATURE INFANTS^{1, 2}

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AND RUTH HARE

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INTRODUCTION

Our present knowledge of water and electrolyte metabolism in young infants is derived almost wholly from balance studies. The high degree of regulation described by such studies is effected by renal mechanisms which we are attempting to define. In any study of kidney function it is necessary to select certain criteria for evaluating the observed data. In young premature infants, this problem of selection is made difficult by the possibility that the substances commonly followed in adult studies may be handled differently either by the immature kidney as such or by the immature organism as a whole. The urea clearance is probably the criterion most often employed in clinical studies of the adult subject. Its use and significance in the infant are less well established. Some studies (1, 2) have suggested that the urea clearance in infants, as in adults, is dependent on urine flow; others (3, 4) have claimed that the two values are independent in infants, and that this represents a functional difference between the immature and the mature kidney.

Another, and perhaps more useful criterion in adult studies has been the clearance of inulin, now well established as a measure of glomerular filtration. There is little information about the clearance of inulin in the infant; it has been suggested that in infants this clearance, although independent of the plasma level of inulin, as in the adult, differs in that it is dependent on rate of urine flow (1, 5).

We considered that simultaneous clearances of inulin and urea at various rates of urine flow would

serve not only to establish a criterion of kidney function in the infants, but might help in the interpretation of previous work where only urea was studied. If inulin clearances measure glomerular filtration rate in the young infant, such studies would in addition yield information on the renal mechanism for water excretion.

METHODS

Clearances were measured in 21 well, female, premature infants, aged three to 28 days, whose birth weights ranged from 1740 to 2480 Gm. and whose weights at the times of observation were 2060 to 2400 Gm. Their surface areas, calculated by the formula $5.188 \times \text{Wt.}^{.75}$ (6), were 0.163 to 0.178 sq. M. A minimum of four and a maximum of 21 clearance periods were measured in any one infant. All observations were made in the air-conditioned metabolism unit in which the temperature and humidity were maintained at 26° C. and 60 per cent respectively.

Low rates of urine flow were produced by withholding feedings and water for varying periods of time up to 12 hours, and by using single injections of inulin rather than a continuous infusion. High urine flows were produced by giving water and feedings by mouth or by the intravenous infusion of 25 per cent mannitol, 0.9 per cent NaCl, 5 or 10 per cent dextrose, singly or in combination. Tables I and Ia show the protocols and data for clearances done on falling plasma levels after single or repeated injections. Table Ib includes similar data for clearances done using the usual priming and sustaining technic (7).

Urine was collected through an indwelling catheter, and the bladder was completely emptied by blowing out with air. The largest multi-holed soft rubber catheter that could be introduced with very slight pressure, usually a size 10 or 12 French catheter, was used and no leakage was observed even during periods of marked straining. Twenty thousand units of penicillin were given intramuscularly immediately and 12 hours after each clearance study. There was never any evidence of local tissue reaction or bladder infection from the catheterization.

In the earlier observations, the urine was collected in 10- or 25-ml. graduated cylinders calibrated in 0.1 or 0.2 ml., and the volumes read directly. Later, the urine was

¹ Aided by a grant from the U. S. Public Health Service. Mannitol and para-aminohippurate were supplied by Sharp & Dohme, Inc.

² Presented in part before the American Physiological Society, Atlantic City, N. J., March 19, 1948 (Fed. Proc., 1948, 7, 5), and before the Society for Pediatric Research, Atlantic City, N. J., May 4, 1948.

TABLE I
Protocols and data on urica and inulin clearances in premature infants

Subject	Total time (min.)	Urine vol. ml./min.	INULIN		UREA N		INULIN U/p		Subject	Total time (min.)	Urine vol. ml./min.	INULIN		UREA N		INULIN U/p
			Plasma conc. mg./ml.	Clearance ml./min.	Plasma conc. mg./ml.	Clearance ml./min.						Plasma conc. mg./ml.	Clearance ml./min.	Plasma conc. mg./ml.	Clearance ml./min.	
RA*	0-90	.013			.100	0.12			ML	0-42	.086			.168	2.29	
	90-124	.103			.107	1.01				42-57	.355			.168	3.26	
	131		Injection of Mannitol intravenously							57	.069					
	124-153	.224			.111	1.33				57-105	.061			.137		
	153-209	.218			.112	1.59				105-145	.108	.552	6.27	.168	1.10	58.0
	209-263	.289			.110	1.99				145-177	.153	.422	4.15	.168	2.61	27.1
	263-335	.264			.116	2.09				177-213	.089	.351	3.22	.168	1.66	36.2
	335-390	.102			.122	0.87				213-276	.033	.278	3.92	.168	0.97	118.8
	390-448	.345			.122	1.88				276-312	.047			.168	1.62	
										312-327	.056	.507	7.32	.168	2.97	85.1
FA	0-60	.047			.131	2.37			MR	0-58	.038			.207	0.81	
	60-121	.246			.150	3.16				58-114	.031			.207	0.95	
	162		Injection of Mannitol intravenously							114-172	.043			.203	0.87	
	121-186	.172			.148	2.13				172-236	.042			.212	0.92	
	186-242	.093			.147	1.98				236-293	.035			.217	0.67	
	242-296	.089			.145	2.12				293-344	.046			.215	0.87	
	296-343	.245			.136	3.42				344-418	.144			.213	1.94	
	343-404	.082			.130	1.92				418-452	.104			.211	1.75	
	404-460	.214			.127	2.87				452-472	.208			.209	4.03	
										472-485	.175			.209	4.24	
CU*	0-45	.049			.085	0.63	***		BG "A" **	0-60	.143			.217	2.53	***
	45-81	.084			.085	1.02	---			60-107	.160			.225	2.50	---
	81-121	.099			.097	1.96				107-177	.208			.222	2.96	
	121-186	.093			.097	2.03				177		Injection of Mannitol intravenously				
	186-242	.081			.096	1.65				177-227	.311			.218	3.75	
	242-296	.054			.095	0.95				227-284	.274			.219	3.01	
	296-343	.059			.093	0.91				284-315	.400			.206	3.26	
										315-349	.170			.205	2.16	
										349-408	.229			.212	2.69	
										408-457	.531			.225	3.52	
ST	0-85	.034			.181	0.57	---		SG "A" **	0-60	.143			.217	2.53	---
	85-134	.145			.181	2.08	---			60-107	.160			.225	2.50	---
	134-144		Injection of Mannitol intravenously							107-227	.311			.218	3.75	
	144-196	.173			.245	1.45				227-284	.274			.219	3.01	
	196-242	.211			.246	2.26				284-315	.400			.206	3.26	
	242-296	.082			.247	0.97				315-349	.170			.205	2.16	
	296-343	.074			.252	1.12				349-408	.229			.212	2.69	
	343-404	.228			.266	1.38				408-457	.531			.225	3.52	
	404-460	.416			.280	3.15										

* Negro
 ** Twin
 *** Control periods on preceding day

3.0 ml. of 10 per cent inulin or 2.0 ml. of 25 per cent mannitol per kilogram body weight were injected the first time either substance was given. Subsequent injections of inulin consisted of 1.5 ml. of a 10 per cent solution per kilogram body weight.

allowed to drain into 50-ml. volumetric flasks, diluted to the mark from a 50-ml. burette and the urine volume calculated by difference. The latter method is more accurate and the immediate dilution of the sample prevents precipitation of inulin or mannitol.

The large scalp veins were found most convenient for drawing blood samples for analysis, since the desired 2 ml. could be obtained without interfering with the infusion. The plasma was separated and the proteins precipitated within two hours.

The dosage of inulin, mannitol and para-aminohippurate (PAH) for individual infants was calculated on the basis of weight and expected clearance. When low urine flows were desired, single injections were used, and the clearances measured during the fall in plasma concentration. When a constant infusion was given, the concentration of the substances in the infusion fluid was varied; the total volume and rate of infusion were the same. The infusion was given through a 22-gauge needle into an ankle, foot or hand vein. The rate was controlled by a Shannon-Bradley clamp³ and measured by a pipette-manometer

³ Available from The Harvard Apparatus Co., Inc., Dover, Mass.

arrangement suggested by White and Findley (8). Figure 1 shows that when the rubber tubing is clamped at B, the rate of fall of the fluid in A, a graduated 5-ml. pipette, may be timed and the rate of infusion regulated by adjustment of the tunnel clamp at F. After calibration with the manometer, the rate of infusion may be estimated in the usual manner by counting drops per unit time at the drip-bulb, C, which also permits observation of the course of the infusion. The T-tube at E allows rapid drainage for replacement of the infusion solution. A filter is inserted at D.

Chemical methods. Mannitol was determined in plasma and urine by the method of Corcoran and Page (9); inulin by a modification⁴ of the method of Hubbard and Loomis (11); and para-aminohippurate by the method of Marshall as described by Goldring and Chasis (7). Urea was determined by the micro-diffusion method of Conway (12), and clearances of NH₂ plus urea were calculated. In some cases, urea was also determined by the method of Archibald (13), and the clearances calculated from

⁴ The acid mixture used by Harrison (10) for the hydrolysis of inulin was substituted for the acid-alcohol mixture employed by Hubbard and Loomis.

TABLE IA

Subject	Total time (min.)	Urine vol. (ml./min.)	INULIN		UREA N		INULIN U/P	Subject	Total time (min.)	Urine vol. (ml./min.)	INULIN		UREA N		INULIN U/P
			Plasma conc. (mg./dl.)	Clearance (ml./min.)	Plasma conc. (mg./dl.)	Clearance (ml./min.)					Plasma conc. (mg./dl.)	Clearance (ml./min.)	Plasma conc. (mg./dl.)	Clearance (ml./min.)	
SW Age 13 days Wt. 2320 Gm. Ht. - S.A. .173m ²	0-48	.050			.221	1.56	***	MA Age 21 days Wt. 2140 Gm. Ht. 45 cm. S.A. .163m ²	0-57	.337			.210	2.84	***
	48-73	.062			.221	2.27			57-92	.544			.210	2.98	
	73-111	.103	Injection of Mannitol intravenously						15		Injection of Mannitol and NaSCN intravenously				
	0-15	.143			.234	1.32			0-15	.203			.211	2.50	
	52-96	.209			.233	2.46			35-85	.131			.211	1.98	
	143				.233	2.46			135-145	.144			.215	2.38	
	96-156	.243	42 ml. of 0.9% NaCl subcutaneously						145-209	.117			.206	2.16	
	156-216	.243			.236	1.45			209-268	.082			.199	1.93	
	216-262	.248			.241	2.77			268-325	.105			.206	2.29	
ST Age 13 days Wt. 2380 Gm. Ht. - S.A. .177m ²	0-59	.051			.238	1.53	***	KE Age 21 days Wt. 2240 Gm. Ht. 47 cm. S.A. .169m ²	0-19	.059			.256	1.54	
	59-94	.182			.238	1.46			30		Injection of Inulin intravenously				
	16		Injection of Mannitol intravenously						19-82	.103			.253	2.26	
	0-19	.051			.236	0.63			82-108	.132	.470	6.02	.248	2.89	
	39-89	.100			.227	0.89			108-138	.210	.350	5.20	.245	2.95	45.5
	89-152	.152			.215	2.20			138-162	.263	.300	4.10	.242	1.24	66.7
	152-209	.160			.220	1.86			162-209	.088	.260	6.15	.238	2.38	70.0
	209-260	.137			.225	2.66			209-246	.142	.228	4.43	.234	2.55	31.1
	260-308	.221			.214	2.66			252		Injection of Inulin intravenously				
									246-302	.064			.230	1.62	
GR Age 18 days Wt. 2370 Gm. Ht. 48 cm. S.A. .176m ²	0-42	.355			.243	1.52	***	BO "B" ** Age 23 days Wt. 2060 Gm. Ht. 46 cm. S.A. .159m ²	36		Injection of Mannitol intravenously				
	42-84	.045			.243	0.80			0-57	.109			.238	2.15	
	84-126	.060			.243	1.26			57-115	.066			.242	0.78	
	126-168	.055			.243	1.78			115-173	.278			.229	2.79	
	168-210	.097			.247	0.91			173-210	.305			.214	1.07	
	0-33	.112			.239	1.24			230-287	.151			.215	1.67	
	33-71	.242			.239	2.84									
	71-104	.114			.239	2.10									
	104-132	.096			.239	2.73									
			Injection of Inulin intravenously												
CO Age 19 days Wt. 2260 Gm. Ht. 45 cm. S.A. .170m ²	132-189	.061			.236	1.80		KE Age 28 days Wt. 2380 Gm. Ht. - S.A. .177m ²	0-30	.044			.168	1.20	
	189-249	.052	.168	3.99	.230	1.72			30-64	.239	Injection of Mannitol and Inulin intravenously				
	249-309	.060	.254	4.25	.224	2.02	76.3		64-115	.671			.163	3.73	
	309-351	.045	.186	3.49	.220	1.53	70.2		115-150	.206	.456	4.84	.160	2.67	23.5
									150-181	.355	.343	6.52	.157	4.39	18.4
	351-426	.100			.216	2.30	76.5		181-209	.225	.282	5.38	.156	3.19	23.9
	426-465	.135	.321	4.02	.215	2.93	29.5		209-241	.116	.232	5.39	.154	2.39	11.0
	465-507	.121	.240	4.21	.218	2.91	34.4		241-271	.220	.196	6.33	.152	3.93	28.8
	507-535	.175	.190	4.11	.221	3.35	23.2		271-285	.477	.173	6.20	.151	4.51	13.0
	535-552	.090	.154	3.25	.224	1.91	21.3		285-300	.230	.160	5.45	.151	3.70	23.7
	552-601	.219	.122	4.76	.228	3.03									

** Twin
*** Control periods on preceding day

these values agreed well with those of the Conway method. No preservatives were used in the urine. In the few instances in which determinations were not done on the day of collection, the sterile catheterized samples were stored in the refrigerator in sterile flasks. Determinations on samples stored in this way showed no change from the original value within 24 hours.

All determinations were done in duplicate, and usually required a total of 1 ml. of plasma. When colorimetric methods were used, a calibration curve was run with each group of unknowns and reagent blanks were used for center settings.

Pre-injection samples of plasma and urine were used for blank determinations in all clearances; the urine blanks were calculated and subtracted as milligrams per minute to allow for variations in the rate of urine flow. The values for plasma concentrations of all substances were plotted on the logarithmic scale against time on the linear scale of semi-logarithm paper. The plasma concentrations interpolated at the mid-points of the periods were used in the calculation of the clearances. All clearances were calculated as UV/P. Since the relation of the clearances in young infants to surface area, body weight, metabolic rate, age or other standards of reference

has not been fully established, we have presented only the uncorrected values.

RESULTS

Values for 229 urea clearances and 110 inulin clearances with rates of urine flow in 23 series of observations on 21 subjects are given in Table I. Graphic analyses of these data are shown in Figures 2, 3, 4 and 5. In Figure 2, the influence of urine flow on urea and inulin clearances is shown. At urine flows below 0.1 ml. per minute the urea clearances were less than 3.0 ml. per minute and were markedly below the inulin clearances so that the symbols representing the two separate completely. At urine flows above 0.4 ml. per minute, on the other hand, there was considerable overlapping. Analysis of the data from only those periods when simultaneous inulin and urea clearances were measured is shown in Figure 3. Here, a slight increase in inulin clearance with increasing

TABLE 1b

Protocols and data on urea and insulin clearances in premature infants

Subject	Total time (min.)	Urine vol. ml./min.	INULIN		UREA N		INULIN U/P	Subject	Total time (min.)	Urine vol. ml./min.	INULIN		UREA N		INULIN U/P
			Plasma conc. mg./dl.	Clearance ml./min.	Plasma conc. mg./dl.	Clearance ml./min.					Plasma conc. mg./dl.	Clearance ml./min.	Plasma conc. mg./dl.	Clearance ml./min.	
BA "A" ** Age 3 days Wt. 2130 Gm. Ht. 46 cm. S.A. .163M ²	0-60	.042			.147	.90		PI "A" ** Age 12 days Wt. 2320 Gm. Ht. 47 cm. S.A. .173M ²	0-37	.054			.216	1.71	
	60-90	.082			.139	1.56			37-70	.167			.209	3.41	
	90-110	.064			.132	1.00			70-84	.339	.506	5.87	.204	3.51	17.3
	110-130	.050			.128	0.92			84-98	.643	.488	7.11	.201	5.15	11.1
	130-150	.076	.390	2.77	.124	1.39	36.4		98-112	1.086	.472	6.78	.198	5.00	6.2
	150-170	.213	.408	3.68	.120	2.35	17.3		112-144		.505	5.81	.189	3.46	11.5
	170-190	.296	.432	3.29	.117	2.40	11.1		144-158		.412	6.97	.180	4.48	16.9
	203								158-172		.525	6.56	.175	4.73	12.7
	190-220	.357	.491	2.79	.113	2.23	7.8		172-186		.695	7.04	.171	5.12	10.1
	220-240	.355	.521	3.45	.110	2.47	9.7								
	240-260	.580	.530	3.45	.108	2.51	6.0								
	260-270	.276	.538	4.96	.107	1.72	18.0								
GU Age 4 days Wt. 2276 Gm. Ht. 46 cm. S.A. .171M ²	0-28	.014						MA Age 12 days Wt. 2190 Gm. Ht. 46 cm. S.A. .166M ²	0-30	.147			.281	1.79	
	31								30-88	.095			.281	1.60	
	28-60	.139					4.0		88-108	.305	.687	4.35	.266	3.37	14.3
	60-74	.179	.469	6.03			6.3		108-125	.616	.640	4.14	.260	3.55	6.7
	74-88	.244	.453	4.34			5.8		125-140	.570	.606	4.37	.254	3.58	7.7
	88-102	.450	.441	5.83					150						
	114								140-170	.311	.581	3.94	.245	2.87	12.7
	102-148	.535	.428	4.91			4.9		170-184	.311	.581	3.32	.242	2.67	10.7
	148-162	.546	.424	5.66			5.7		184-198	.443	.585	3.93	.242	3.49	8.9
	162-176	.734	.424	5.99			6.0		198-212	.648	.588	4.35	.242	3.91	6.7
	176-190	1.130	.424	5.54			5.5								
RA "B" ** Age 9 days Wt. 2380 Gm. Ht. 47.5 cm. S.A. .177M ²	0-31	.122			.200	2.82		RO Age 13 days Wt. 2164 Gm. Ht. 45 cm. S.A. .165M ²	0-67	.195			.234	1.88	
	31-100	.080			.194	1.60			67-133	.218			.224	2.29	
	100-116	.243	.637	4.73	.188	3.42	19.4		133-153	.290	.476	4.28	.212	3.05	14.8
	116-130	.463	.605	5.11	.186	4.19	11.0		153-167	.618	.466	4.78	.205	3.28	7.7
	130-140	.538	.581	4.27	.185	3.50	7.9		167-185	.272	.455	3.34	.200	3.28	12.3
	140-149	.433	.564	3.99	.183	3.07	9.2		185-201	.400	.430	5.20	.195	1.66	13.0
	156								201-213	.500	.417	4.18	.193	3.23	8.4
	149-177	.243	.530	3.77	.178	2.50	15.5								
	177-194	.241	.498	4.38	.170	3.08	18.2								
	194-205	.391	.488	5.33	.166	4.23	13.6								
	205-215	.560	.481	5.18	.163	4.39	9.3								
PI "B" ** Age 9 days Wt. 2216 Gm. Ht. 47 cm. S.A. .168M ²	0-46	.116			.194	1.98		PA Age 13 days Wt. 2164 Gm. Ht. 45 cm. S.A. .164M ²	0-20	.130			.216	2.02	
	46-86	.296			.194	3.21			20-60	.103			.214	1.41	
	86-100	.287	.390	6.13	.192	2.92	21.4		60-80	.092	.629	2.26	.210	1.37	24.5
	100-114	.218	.410	5.95	.191	3.48	27.3		80-100	.151	.628	3.69	.208	2.46	24.1
	114-128	.286	.440	5.34	.190	3.52	18.7		100-120	.299	.623	3.10	.206	2.34	10.5
	139								126						
	128-160	.401	.456	5.00	.189	3.66	12.5		120-150	.154	.611	2.78	.201	1.89	18.1
	160-174	.466	.431	5.55	.182	4.01	11.9		150-172	.194	.607	3.38	.195	2.40	17.4
	174-188	.432	.414	5.15	.186	3.68	11.9		172-184	.315	.618	4.09	.191	3.19	13.0
	188-202	.507	.402	5.30	.185	3.64	10.5		184-196	.353	.629	3.74	.188	3.13	10.6

** Twin

Priming injections consisted of 2.0 ml. of 10 per cent inulin, 1.0 ml. of 25 per cent mannitol, and 0.025 ml. of 20 per cent para-aminohippurate per kilogram body weight. Sustaining infusions were given at the rate of 0.5 ml. per minute and the concentrations of the substances varied on the basis of expected plasma levels and clearances.

rate of urine flow becomes apparent; but for a given increase in urine flow, particularly at low rates, the urea clearances increased much more than the inulin clearances.

In most instances the course of a diuresis was followed until the urine flow returned to the original rate; four examples are shown in Figure 4. In one, the urea clearance was higher during periods of increasing urine flow than during periods of decreasing flow when the rates were the same. This is the effect found by Shannon (14) in the dog and by Chasis and Smith (15), in man. The other three examples, which are more representative, showed no such relationship.

The foregoing analyses have considered only the excretion of water and urea. Figure 5 shows the relationship of urea reabsorption expressed by the urea/inulin clearance ratio to water reabsorption expressed by the inulin U/P ratio (14). That there is a high degree of correlation between the

reabsorption of these substances is obvious, as was the correlation between their excretions shown in Figure 4. A similar relationship between the tubular reabsorption of urea and of water has been described in normal adult human subjects and in patients with glomerulonephritis and with hypertensive disease (15). The relationships in the various groups of subjects, while similar, are not identical; in fact, the data from the premature infants are more comparable to those from patients with glomerulonephritis or hypertensive disease, and are decidedly different from those from normal adults. A possible interpretation is that there is a back-diffusion or reabsorption of inulin in premature infants. This explanation seems unlikely because a constant clearance of inulin may be maintained in the presence of wide variations in water reabsorption. Furthermore, the mannitol/inulin clearance ratio is the same as in adult subjects in whom inulin is certainly a measure of glomerular

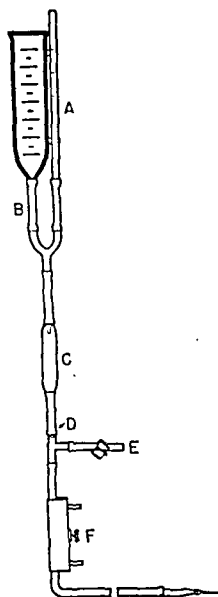


FIG. 1. DIAGRAM OF THE INFUSION APPARATUS

A, a 5-ml. Mohr pipette; B, rubber tubing from reservoir; C, drip-bulb; D, filter; E, side-arm for draining reservoir; F, Shannon-Bradley tunnel clamp.

filtration. Berger, *et al.* (16), have found that the infusion of mannitol depresses the clearance of inulin in adults; Table II shows that in our observations on infants no such effect was observed. There are two factors, however, which may account for the difference in the two series: the difference in age of the subjects and the fact that the plasma levels of mannitol in the infants were around 50 mg. per 100 ml., whereas in the adults they were usually between 125 and 150 mg. per 100 ml. (17).

TABLE II

Inulin clearances before and after administration of mannitol

	INULIN CLEARANCES*	
	PRIOR TO MANNITOL INFUSION	DURING MANNITOL INFUSION
MEAN VALUE ML./MIN.	4.33	4.47
NUMBER OF OBSERVATIONS	27	28
NUMBER OF SUBJECTS	5	5

* AGE 8-21 Days Wt. 2164-2400 Gm. S.A. 0.165-0.178 M²

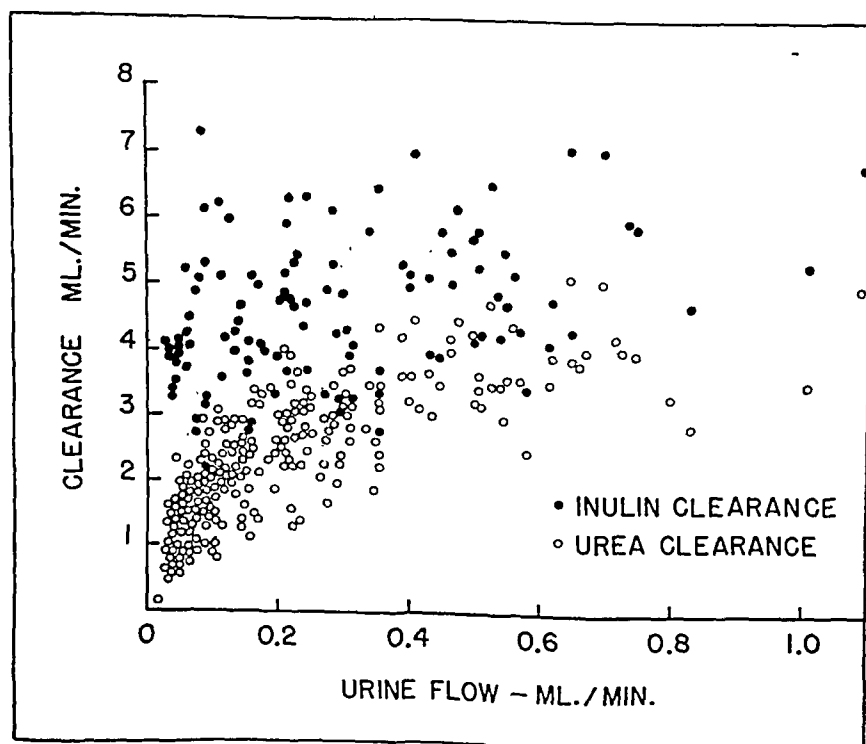


FIG. 2. ABSOLUTE VALUES FOR UREA AND INULIN CLEARANCES IN PREMATURE INFANTS PLOTTED AGAINST RATES OF URINE FLOW

The clearance of any substance excreted solely by glomerular filtration should be (a) independent of plasma concentration; (b) independent of urine flow; (c) not influenced by the excretion of any known substance; (d) identical with that of other substances known to measure glomerular filtration. The clearance of inulin in these premature infants satisfies the first three of these criteria: it is independent of plasma concentrations from 12 to 68 mg. per 100 ml.; it is independent of rates of urine flow from .032 to 1.13 ml. per minute; it is not influenced by any substance used in these studies, *i.e.*, glucose, mannitol or PAH. The direct comparison of inulin clearance with any established measure of glomerular filtration in the infant is impossible, since no substance has been proved to measure glomerular filtration in the infant. However, the average mannitol/inulin clearance ratio in these infants is 0.883 which is almost identical with

that of 0.902 found by Corcoran and Page for adults, in whom inulin is a proved measure of glomerular filtration. Inulin is, therefore, probably excreted solely by the glomerulus in infants.

The inulin clearance in premature infants is low compared to that of adults, not only as an absolute value but when expressed in terms of surface area. The average clearance for this group of premature infants corrected for surface area is less than 50 ml. per 1.73 sq. M, whereas the average value is 117 ml. (7) for the adult female. This low rate of glomerular filtration in young infants has been observed previously (1, 4, 5, 18-20)

It is obvious from the preceding data that the clearance of inulin is a valuable aid in studying the kidney function of premature infants. On the other hand, urea clearance alone is without meaning; it can be interpreted only when glomerular filtration is known. This is because it is de-

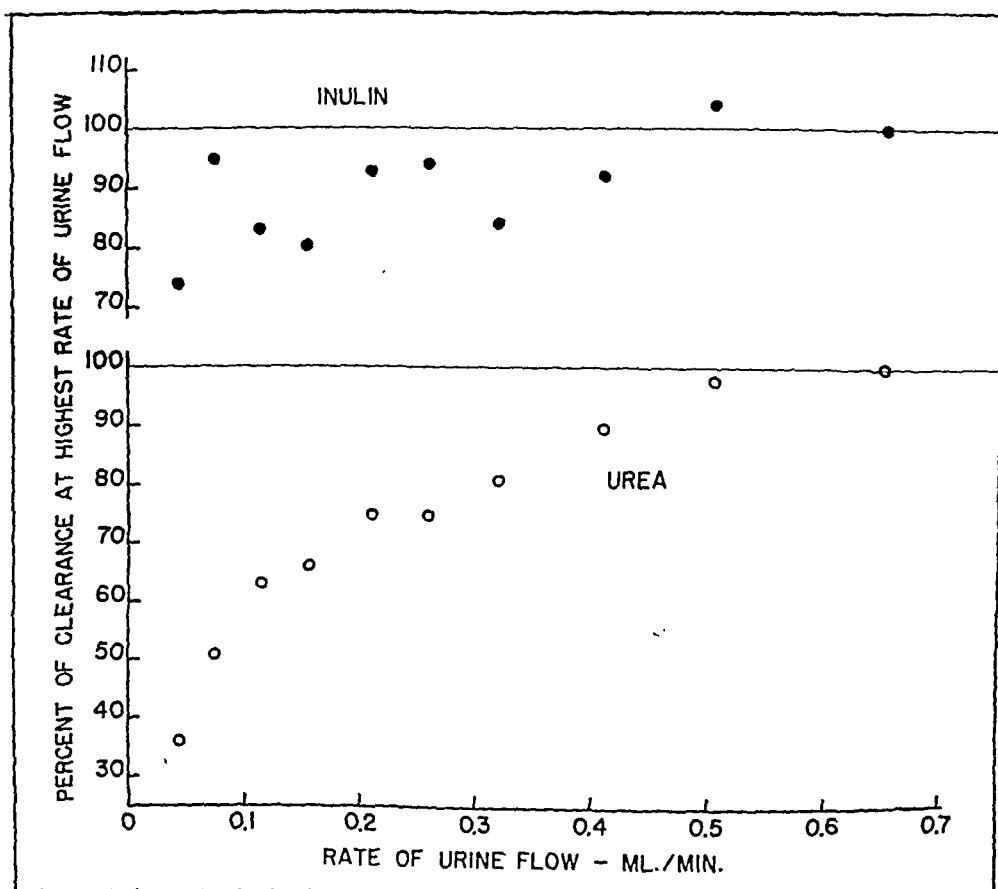


FIG. 3. CHANGES IN INULIN AND UREA CLEARANCES WITH CHANGES IN RATE OF URINE FLOW IN PREMATURE INFANTS

These data were calculated from 100 periods during which inulin and urea clearances were measured simultaneously. Each point represents an average of ten periods. The clearances are expressed as per cent of the average clearance at the highest average rate of urine flow observed (0.6 ml. per minute).

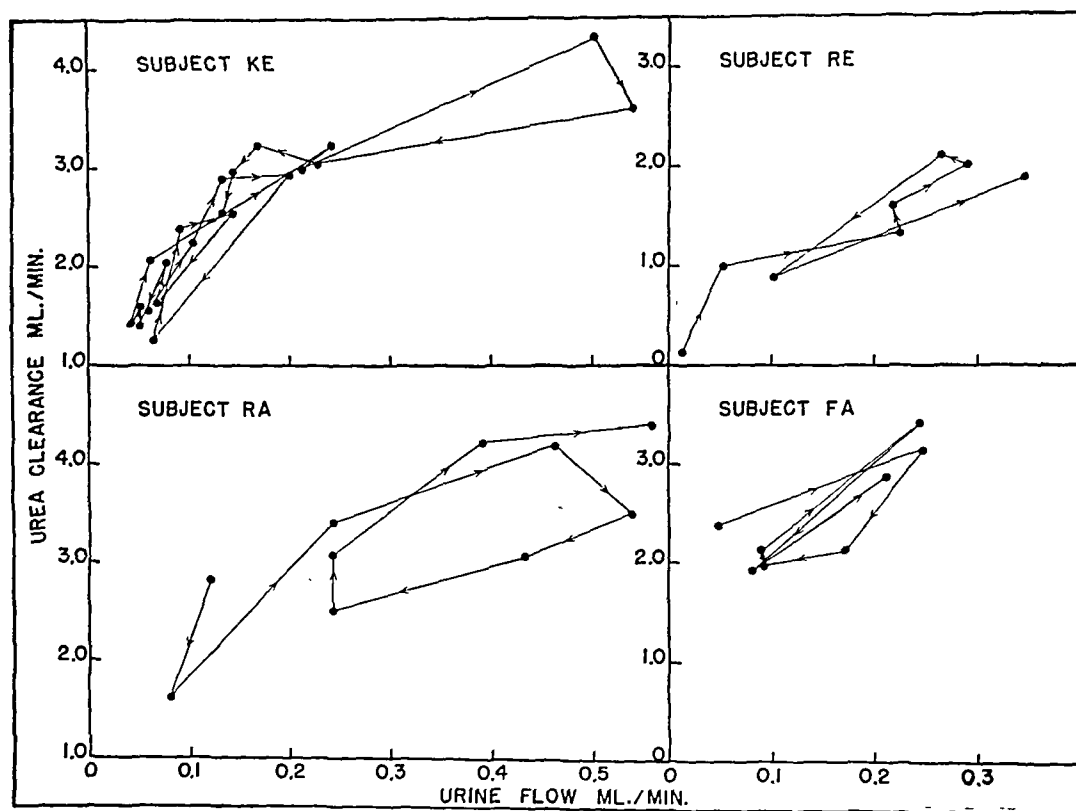


FIG. 4. RELATION OF UREA CLEARANCE TO RATE OF URINE FLOW IN INDIVIDUAL SUBJECTS
The arrows indicate sequence of observations.

pendent upon several different independent variables: glomerular filtration, tubular transfer of water and rate of change of urine flow. Urea clearance is, therefore, dependent upon both glomerular and tubular activity, and measures no specific renal function. If urine flow changes at a rapid rate, the relation of urea clearance to urine flow is occasionally disturbed. Shannon (14) has described abrupt increases in urea clearance during the onset of a diuresis ("exaltation"), and we find marked depressions in the urea clearance with rapid decreases in urine flow in several instances; the two points at a U/P ratio of 20 and a urea/inulin clearance ratio of 0.3 to 0.4 in Figure 5 were examples of this. In general, it is true that urea clearances vary directly with rate of urine flow, but the quantitative change in clearance is unpredictable: in Figure 5, at an inulin U/P ratio of 20, the urea clearance varied from 35 to 80 per cent of the inulin clearance. The variation in an individual experiment is shown in Figure 4. It has been reported that infants showed no consistent change in urea clearance with urine flow (3, 4),

but the range of observations in those studies was narrow, and if their data are plotted on our graphs, the points fall on our curves.

If inulin does measure glomerular filtration, then the inulin U/P ratio measures the tubular reabsorption of water. After moderate water deprivation inulin U/P ratios as high as 227 were found in premature infants. This indicates that, in the immature kidney, water is conserved when dehydration occurs without electrolyte loss, chiefly by increased tubular reabsorption of water rather than by decreased glomerular filtration. This mechanism of water conservation is comparable to that of the adult. Measurements of osmotic pressure and of specific gravity have not given a true evaluation of water conservation in infants (21-23). This may be explained by the fact that these values are determined not only by the tubular reabsorption of water, but by the reabsorption of solutes as well.

Tubular reabsorption of water is believed to be under the control of the anti-diuretic hormone of the posterior pituitary. Finding a high capacity

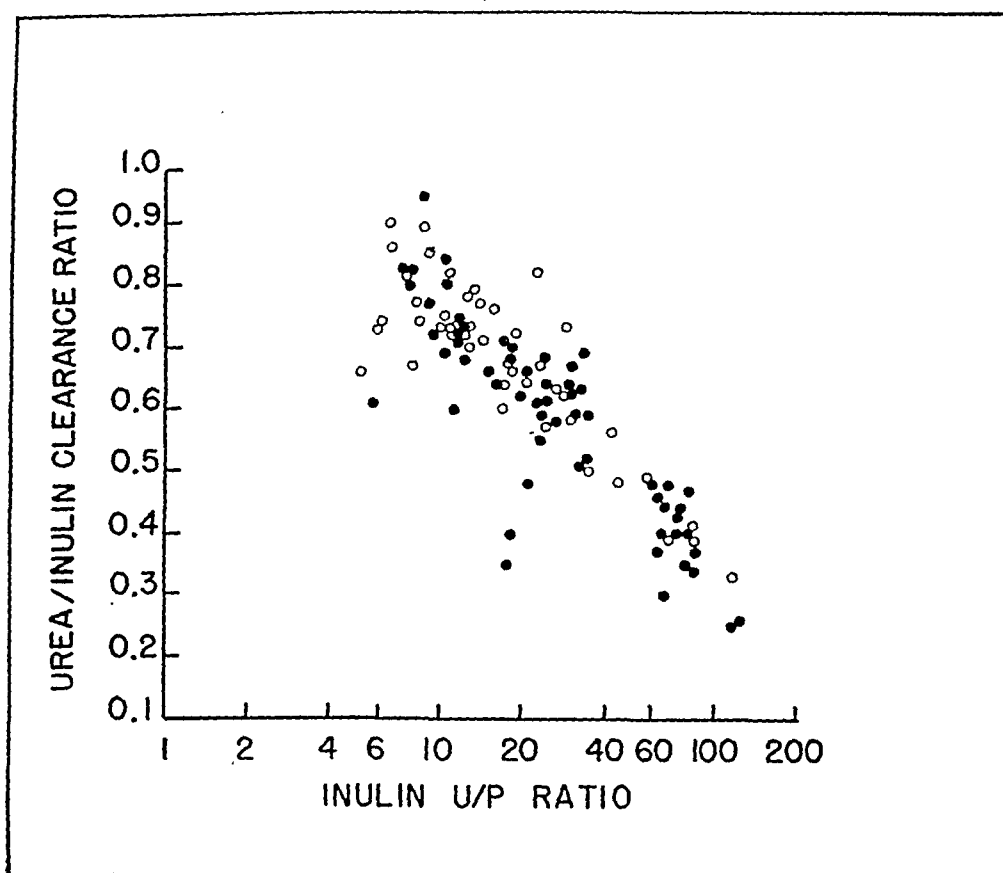


FIG. 5. RELATION OF TUBULAR REABSORPTION OF UREA TO TUBULAR REABSORPTION OF WATER

Inulin U/P ratios are plotted on a logarithmic scale and clearance ratios on a linear scale. The dots represent periods of constant or decreasing urine flow, the circles periods of increasing urine flow.

for water reabsorption by the tubules of the premature infant's kidney conflicts with the finding of Heller (24) that the tubules are relatively insensitive to pitressin. We have, in a preliminary way, put this to the test by more crucial observations than those employed by Heller. A representative observation in a six-day-old, 2200-Gm. premature infant is shown in Figure 6. High inulin U/P ratios were found, as expected, following 16 hours of water deprivation. The infant was then given enough fluid to produce a water diuresis during which the U/P ratios fell as indicated. Pitressin was then infused at the rate of 1 milli-unit per minute and an immediate rise in the U/P ratio followed. These observations indicate a good response of the tubules of the premature infant to anti-diuretic hormone. We cannot, at the present time, explain the finding of higher inulin U/P ratios following water deprivation than during pitressin infusion. This discrepancy apparently does not represent a peculiarity on the part of pre-

mature infants, however, since Taylor, *et al.* (25), measuring urine specific gravity, found the same difference in response in adults.

CONCLUSIONS

1. The renal clearance of inulin in premature infants apparently measures glomerular filtration.
2. The inulin clearances of premature infants are low compared to those of adults on a basis of surface area.
3. Urea clearances of premature infants are also low, but cannot be used in estimating glomerular filtration since they have a variable relationship to inulin clearances. The ratio of urea clearance to inulin clearance is markedly influenced by urine flow.
4. The renal tubules of the premature infant are capable of reabsorbing more than 99 per cent of the water in the glomerular filtrate.
5. The renal tubules of the premature infant are responsive to the pituitary anti-diuretic hormone.

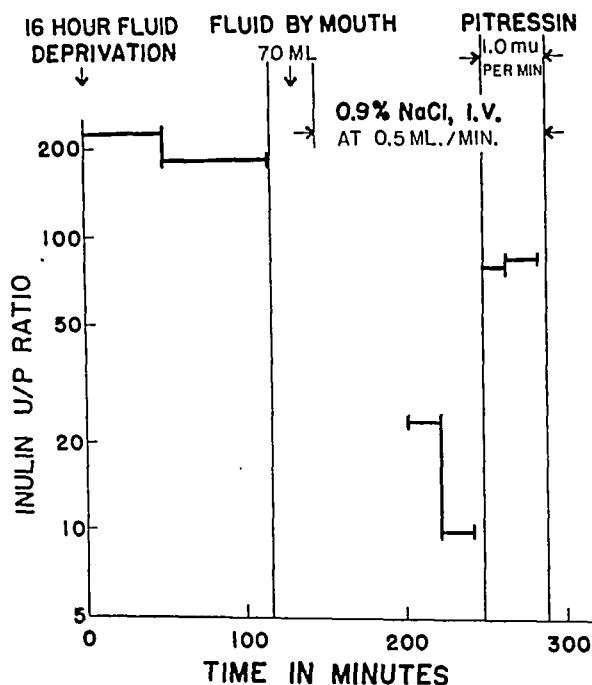


FIG. 6. RENAL RESPONSE AS MEASURED BY INULIN U/P RATIOS OF A PREMATURE INFANT TO WATER DEPRIVATION, TO WATER ADMINISTRATION, AND TO PITRESSIN INFUSION

6. In premature infants, dehydration produced by withholding fluids does not diminish glomerular filtration but results in increased tubular reabsorption of water.

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ESTIMATIONS OF THE DECREASE IN EFFECTIVE BLOOD VOLUME WHEN PRESSURE BREATHING AT SEA LEVEL¹

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Studies conducted in this laboratory and reported to the OSRD (1) as early as August, 1944, drew attention to the value of counter-pressurization of the extremities in preventing collapse when breathing at intermittent pressures of 30 to 40 mm. Hg. Recent reviews of the physiology of pressure breathing (2, 3) comment on the tendency towards circulatory collapse during pressure breathing and suggest that the failure is probably associated with a decrease in effective circulating blood volume. This could be engendered by a pooling of blood in the congested veins of the extremities, by a loss of fluid due to filtration from the circulation under conditions of increased capillary hydrostatic pressure, or by a combination of both mechanisms. Fenn, Otis, and their collaborators (4) have measured the approximate amount of blood trapped in the leg veins during pressure breathing by a technique involving comparisons of a teeter-board and leg plethysmograph data. They conclude that during pressure breathing some 3 per cent of the total blood volume may be sequestered in the legs alone. Barach, *et al.* (3) have calculated the loss of fluid from the circulation during pressure breathing from measurements of changes in the oxygen capacity and the plasma protein concentrations of the blood. In men subjected to continuous pressure breathing at levels of 20 cm. water for periods of less than one hour, the oxygen capacity data indicate a hemoconcentration of about 5 per cent, while the plasma protein data give a calculated fluid loss of 6.5 per cent.

Since both losses are associated with abnormal relations between the intravascular and tissue pressures in the extremities, it is theoretically possible

to decrease these losses by effective counter-pressurization of the extremities. Barach, *et al.* (3) find that fluid loss during pressure breathing can be diminished from 6.5 per cent to 2.3 per cent when the subjects are provided with tight-fitting counter-pressurization pants.

We have measured the extent of fluid loss during continuous pressure breathing at several pressure levels by means of changes in the hematocrit and hemoglobin values. Since the changes in the pressure gradient across the vascular walls in the extremities during pressure breathing is of a magnitude which might lead to distension and possible changes in the permeability of the vascular wall (cf. 5), plasma protein concentrations were determined and the tendency for protein leakage from the circulation calculated.

METHODS

Five male subjects in normal health and of ages ranging from 20 to 30 years were used. They were fitted with a full-face pressure breathing mask and a pressure vest of appropriate size and with tights snugly fitted over the legs and buttocks. The pressure breathing mask was supplied with a Linde exhalation valve and a standard check valve. After 30 minutes of rest in the sitting position, to decrease the effects of postural changes on the blood volume (6), 10-12 cc. blood were taken from a forearm vein after heating the arm for three minutes to avoid stasis. Continuous pressure breathing was then carried out for 30 minutes at pressure levels varying from 20 to 63 mm. Hg, and at the end of this time a second blood sample was taken from the back of the hand after heating to 47-48° C. for three to five minutes. The building in which these experiments were carried out was air-conditioned, and the temperature was held at approximately 23° C.

Hematocrits were determined in duplicate using the Wintrobe technique (7). Hemoglobin estimations were also made in duplicate by use of the acid hematin technique. The color developed was estimated with a Klett-Summerson photoelectric colorimeter. Plasma protein concentrations were estimated in duplicate using the Mehl technique (8). Estimations were made immediately after

¹ This work was performed under contract recommended by the Committee on Medical Research between the OSRD and the University of Southern California. It was reported in July, 1945, to the Committee on Aviation Medicine as Report No. 452.

drawing the blood into heparinized syringes. The results obtained are accurate to the order of plus or minus 1.0–2 per cent. On several occasions, radial arterial blood was taken in place of the heated hand vein specimen.

The per cent fluid loss was approximately from the hematocrit and the hemoglobin data according to the following equations:

$$\text{Fluid loss} = 1 - \frac{\text{Control hemoglobin}}{\text{Experimental hemoglobin}}$$

$$\text{Fluid loss} = 1 - \frac{\text{Control hematocrit}}{\text{Experimental hematocrit}}$$

This calculation assumes an unchanged volume of circulating red blood cells, and therefore can yield only an approximation of the true changes in plasma volume.

Plasma protein leakage was calculated according to the equation presented by Landis and co-workers (5). Each subject was submitted to two or three estimations at each pressure in an attempt to confirm the values obtained. The following experimental combinations were employed: Mask only, mask and vest, and mask and vest with leg counter-pressure.

RESULTS

In Table I and Figure 1, the calculated fluid losses are presented in terms of percentage change in blood volume. Blood volume was adopted as the reference standard since the significance in terms of possible circulatory collapse of a rapid loss of 12 per cent of the blood volume is well recognized. This represents a one-pint transfusion donation from an average blood volume of 5000 cc. When no leg counter-pressure was used, the average pressure required to induce an 8 per cent change in blood volume in 30 minutes was 50 mm. Hg. The use of a jacket did not change this figure appreciably, but leg counter-pressure provided approximately 20 per cent protection. The figures obtained are variable. This may be because the calculation of fluid loss from hematocrit and hemoglobin changes presupposes that the changed readings are due to fluid loss alone. Systemic fluctuations in the red cell count during the experiment will vitiate this assumption and it is probable that the disturbances induced by pressure breathing include such variations in red cell count (6). Because of these and other technical difficulties it was not possible to present consistent evidence in all subjects of the advantage derived from the use of experimental counter-pressurization of the legs. However, the trend toward a lower fluid loss when using leg protection is still apparent. The data for the hemoglobin de-

TABLE I

Pressure mm. Hg	Subject	Runs	% Fl. loss hema- tocrit	% Fl. loss hemo- globin	% Protein in filtrate
A. With mask only					
0	C	1	0.5	1.0	6.7
20	A	4	1.5	2.3	-2.7
20	C	2	1.0	2.5	-0.1
20	Avg.	6	1.3	2.3	-1.8
30	B	1	3.3	5.9	7.2
30	C	1	7.0	2.0	—
30	Avg.	2	5.1	3.9	7.2
B. Mask and vest					
20	D	1	0.0	0.5	—
20	E	2	0.4	2.5	3.2
20	Avg.	3	0.3	1.8	1.1
30	A	1	5.1	8.2	3.3
30	C	1	3.3	4.0	1.1
30	D	3	3.3	3.0	-2.7
30	E	1	3.0	—	-7.4
30	Avg.	6	3.5	4.2	-1.9
40	A	2	6.5	9.3	1.7
40	B	2	5.8	5.0	1.7
40	C	5	6.3	5.9	-1.1
40	Avg.	9	6.2	6.5	0.1
53	B	5	9.0	10.9	2.7
53	C	2	8.7	7.0	1.8
53	Avg.	7	8.9	9.8	2.4
C. Mask, vest and pants					
30	A	2	4.6	7.4	1.1
30	C	1	2.3	3.2	-0.4
30	Avg.	3	3.8	6.0	0.6
40	A	4	3.4	6.0	4.8
40	B	3	2.9	5.9	2.2
40	C	2	5.0	5.3	0.5
40	Avg.	9	3.6	5.8	3.0
53	A	1	5.7	8.0	0.4
53	B	5	7.8	8.2	2.6
53	C	2	5.0	3.9	-0.4
53	Avg.	8	6.8	7.1	1.8
63	B	3	11.5	10.3	2.4
63	C	1	7.4	5.7	0.3
63	Avg.	4	10.5	9.1	1.9

termination (cf. Figure 2) paralleled those for the hematocrits. Although more variable, they confirm the readings obtained and show that there is no consistent change in cell volume. Landis, *et al.* (5) point out that a change in cell volume should lead to a systematic discrepancy between the hemoglobin and hematocrit values. In practice,

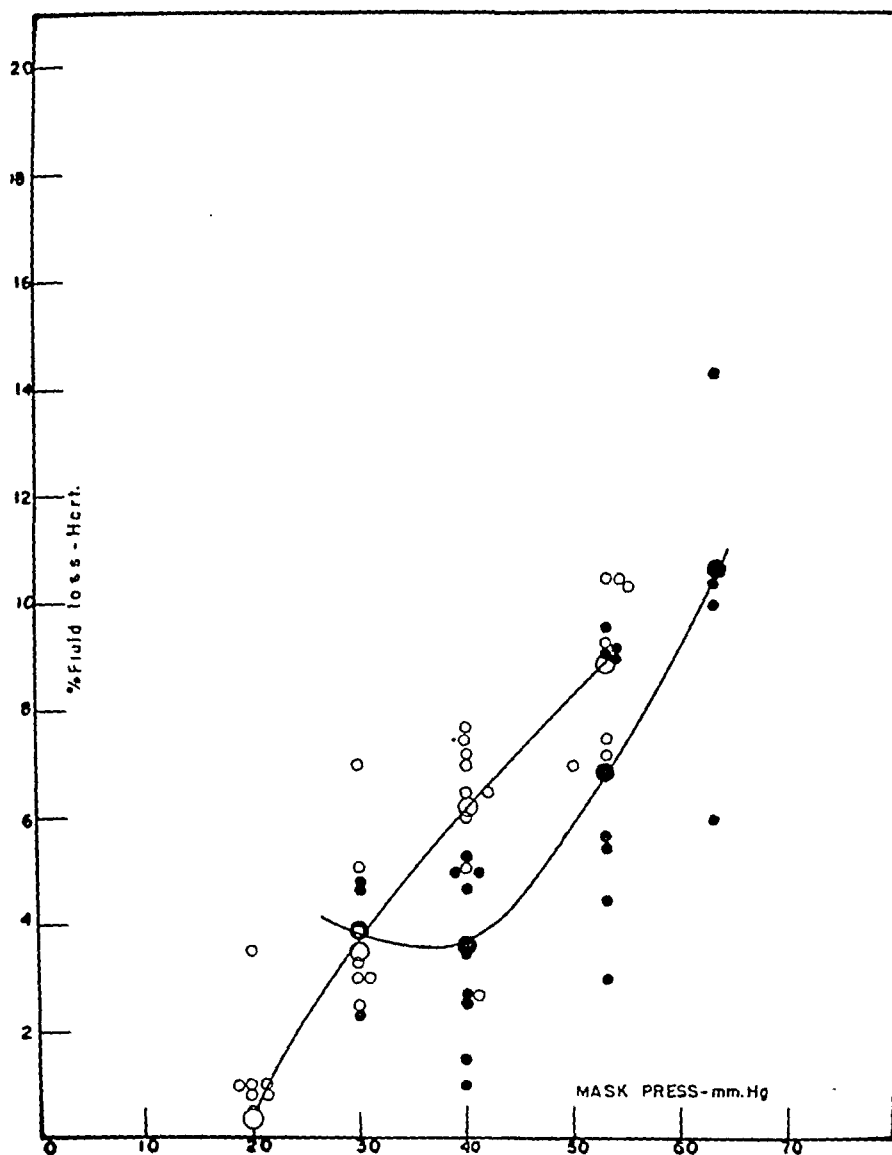


FIG. 1. FLUID LOSS AS A FUNCTION OF MASK PRESSURE

Ordinates: per cent fluid loss calculated from hematocrit data; abscissa: mean mask pressure. Small open circles: individual data for subject wearing mask or mask and vest. Large open circles: average values for mask and mask and vest data at each pressure. Small solid circles: individual data for subjects wearing mask, vest and pants. Large solid circles: average values for subjects wearing mask, vest and pants at each pressure.

the deviations observed were random and therefore probably of experimental origin.

In Table I the calculated protein concentrations of the filtrates are given. There is an increase in protein leakage when the pressure breathing is done at the higher levels. In the case of subject B, the data are moderately constant and are of 2 per cent protein in the filtrate. Subjects A and C give more variable data, including a number of negative values. Henry, *et al.* (9) have set forth

a theoretical basis for the occurrence of these negative values.

Goldschmidt and Light (10) and others (11) have shown that the composition of arterialized hand vein blood does not differ significantly from that of true arterial blood. However, it was thought that in the circumstances of this experiment, the method of obtaining arterialized blood by heating the hand might be associated with some error. Congestion in the exposed unpressurized

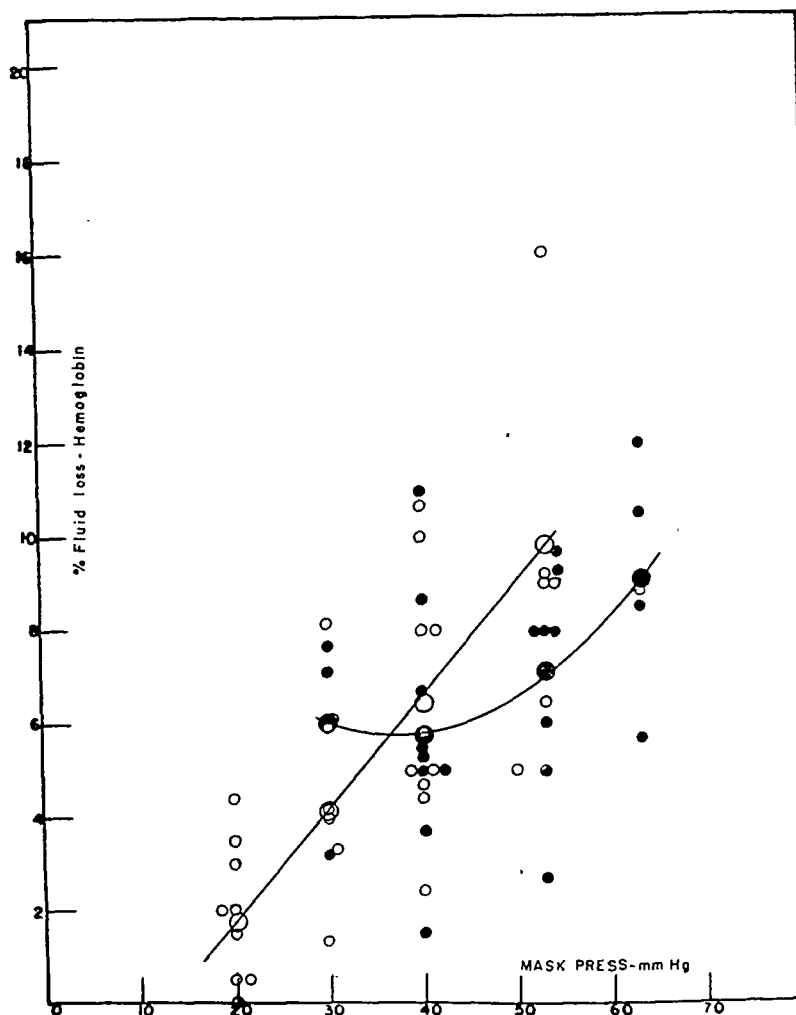


FIG. 2. PER CENT FLUID LOSS AS A FUNCTION OF MASK PRESSURE

Symbols as in Figure 1. Per cent fluid loss calculated on the basis of hemoglobin data.

arms might lead to an increase in local fluid loss and stasis in spite of the vasodilation, thus making the blood sample unrepresentative of the effective circulating blood volume. However, as shown in Table II, checks of arterial blood taken simultaneously with hand vein blood show no significant difference provided there has been adequate heating of the hand.

Figure 3 presents curves for subjects A and C showing the rate at which hemoconcentration develops when pressure breathing with only a vest and mask. The general form is the same in both cases and there is a return to normal within 30 minutes after pressure breathing was stopped.

However, there are interesting differences in detail. In subject A, hemoconcentration proceeded at an approximately uniform rate throughout. In subject C, the rate of development was slower towards the end of the 30-minute period. Waterfield has noted individual variations in the fluid loss due to postural changes (6) and has attempted to correlate them with constitutional differences between the various subjects, including differences between the "firmness" of their tissues. Such inequalities in tissue turgor may also be the reason for the variations noted in the curves for progressive hemoconcentration when pressure breathing.

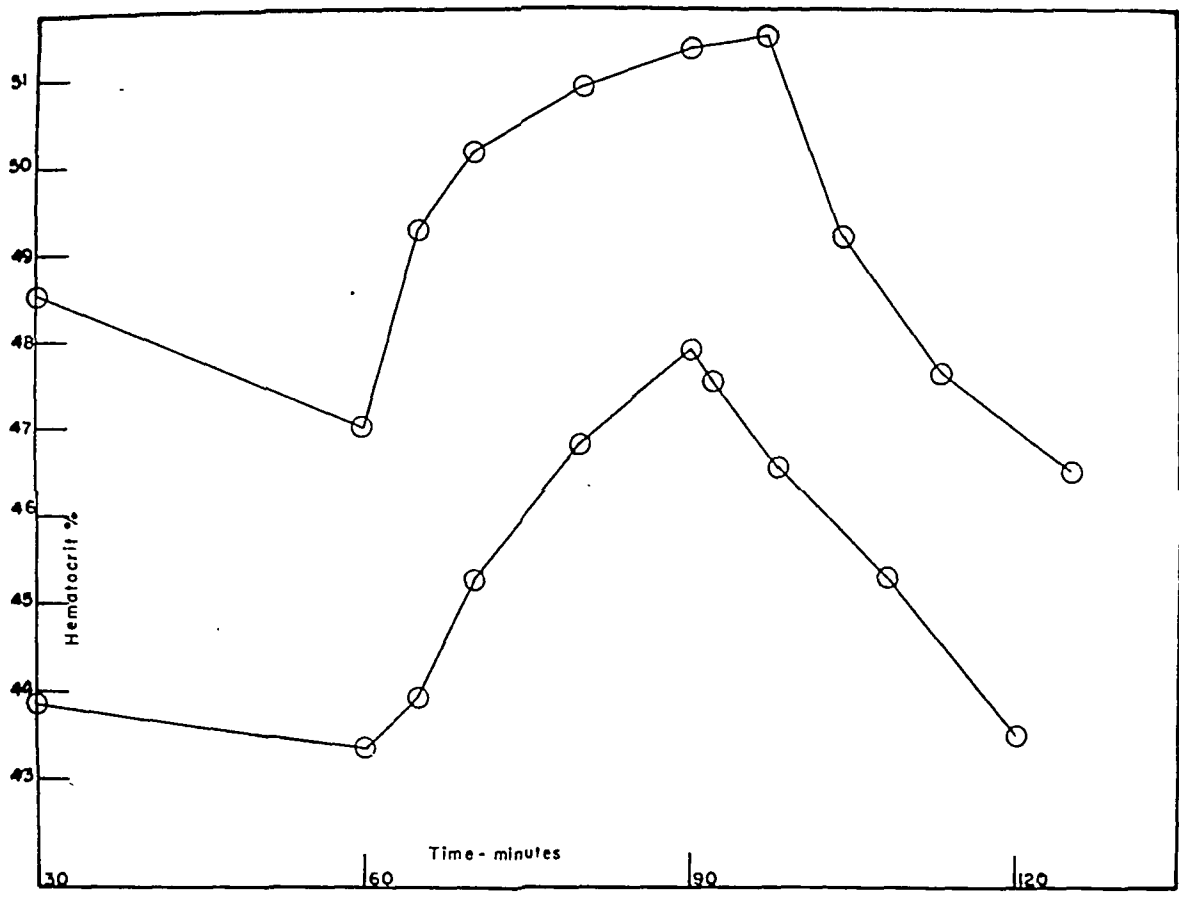


FIG. 3. CHANGES IN HEMATOCRIT VALUE DURING PRESSURE BREATHING FOR TWO SUBJECTS
Upper curve data relative to subject C, lower curve data relative to subject A.

TABLE II
Comparison of simultaneous arterial and arteriovenous blood after pressure breathing for 30 minutes at 50 mm. Hg

Exp.		Hematocrit	Hemoglobin	
			Klett	Grams/100 cc.
1	Arteriovenous	49.2	292	15.5
		49.3	293	
	Arterial	49.1	284	15.1
		49.4	284	
2	Arteriovenous	49.0	267	14.3
		49.2	267	
	Arterial	49.0	268	14.35
		49.0	267	
3	Arteriovenous	50.7	312	16.6
		50.1	315	
	Arterial	50.0	305	16.3
		50.0	312	

DISCUSSION

Fenn, *et al.* (4) have shown that about 300 cc. of blood are displaced into the legs by pressure breath-

ing at 23 mm. Hg. Ebert and Stead (12) showed that the application to one arm and both legs of pressure cuffs inflated to diastolic pressure, *i.e.*, 70-100 mm. Hg for seven to 10 minutes, results in a loss of blood volume of the order of 700 cc. Their data suggest that not more than one-third of this loss was due to the accumulation of fluid in the tissues. The remaining volume change they considered to be due to unchanged blood pooled in the peripheral veins.

A smaller ratio (of the order of 1:1) between the loss due to venous pooling and that due to increase in tissue fluid has been observed by Turner, Newton, and Haynes (13) in their studies of the swelling of the legs in the erect posture. If this lower ratio also applies to the events when pressure breathing, then the frequency with which syncope occurs when breathing at pressures of 50 mm. Hg can be readily explained. At this pressure, the average change in blood volume in the course of 20 to 30 minutes is 8 per cent. This represents a fluid loss of 400 cc. in a subject having the average blood volume. The addition of a fur-

ther 400–500 cc., representing blood pooled in the veins, gives a value of 800–900 cc. for the total loss in effective blood volume. This is enough to bring the average subject to the verge of collapse even when he is not anoxic. Similar estimations would give a total loss of the order of 300–400 cc. for pressures of 30 mm. Hg. This figure might help to account for the frequency with which syncope occurs in the anoxic subject when breathing at this lower pressure with a mask alone.

Objective evidence of the protection against collapse afforded by the use of counter-pressure clothing is given by the average decrease of approximately 20 per cent noted in the fluid loss for pressures in the range 40–60 mm. Hg. It would be desirable to demonstrate that this decrease in fluid loss is accompanied by a reduction in the extent of venous pooling.

SUMMARY

1. Pressure breathing for 30 minutes at 30 mm. Hg at sea level leads, in the average subject, to a calculated loss into the tissues of 4 per cent of the blood volume. Raising the pressure to 53 mm. Hg doubles this fluid loss.
2. The use of a pressure jacket greatly increases comfort but does not decrease the fluid loss significantly. The use of counter-pressure on the legs decreases the fluid loss by about 20 per cent.
3. Counter-pressurization of the limbs is discussed as a method for minimizing the decreases in effective circulating blood volume during pressure breathing.

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RESISTANCE TO THE ACTION OF THE ENDOTOXINS OF ENTERIC BACILLI IN MAN¹

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The somatic or "O" antigens of the gram-negative, enteric bacilli represent the principal toxic and antigenic constituents of these organisms (1, 2). Injected intravenously in man in minute amounts, these antigens produce marked physiological reactions consisting of headache, malaise, chills, fever and granulocytopenia (1). Following repeated injections, the severity of the successive reactions declines until the patient may show no reaction to the injections.

In rabbits injection of these toxic somatic antigens produces similar effects. The decreasing severity of the reactions in the rabbits was shown to be independent of circulating antibody for the antigen injected (2). This "resistance" was also active against certain other somatic antigens which are chemically similar but immunologically distinct (3). Because this resistance is independent of the presence of homologous circulating antibody, the term "tolerance" was suggested (1). The results of further observations on the nature of this tolerance in man are presented here.

MATERIALS AND METHODS

Preparation of antigens. The somatic antigens of *Salmonella typhosa* and *S. schottmuelleri* were prepared by the technique previously described (2). The phenol extraction technique (4) was used to prepare the material from *Shigella dysenteriae*.³ The stock solutions of antigen were prepared in distilled water and further dilutions made in sterile saline for injection.

Methods of administration. The patients selected for study included three normal subjects (KD, GF, MB), two patients with gonorrheal infections (SW, IF) and two with asymptomatic, serological syphilis (ML, SM).

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³ Obtained through the kindness of Dr. Manson Meads.

The group included four males and three females. Four patients (SW, IF, ML and SM) were receiving treatment with crystalline penicillin G. The injection of antigen in from 0.5 to 1 ml. of saline was given into the median basilic vein. Observations of rectal temperature, pulse and respirations were made just before the injection and at hourly intervals thereafter for six hours or until the temperature fell below 100° F. The febrile reaction is expressed in terms of fever units considering one degree Fahrenheit above 100° F. for one hour as one unit of fever.

Serological tests. The "O" agglutinin titers of the serum of each patient for *S. typhosa* (typhoid), *S. schottmuelleri* (paratyphoid B) and *Sh. dysenteriae* (Shiga) were determined before the antigen was given and at intervals thereafter. This test was used as a measure of the antibody response to the somatic antigen since previous studies had indicated that it was an accurate measure of the presence of specific antibody for the somatic antigen injected in rabbits (2) and man (1).

RESULTS

Effective dosage and systemic reactions following injection of antigens. Following their intravenous injection in doses ranging from 0.5 µg. to 2.0 µg. these somatic antigens induced reactions. Within 40–50 minutes, the patients developed severe, throbbing, frontal headaches, began to feel chilly and then had shaking chills and muscular aches. In some instances, they became nauseated and vomited. The temperature rose, reached a peak in about two to three hours and then fell slowly to normal levels in from six to eight hours. The Shiga antigen appeared to be the most toxic, producing severe reactions in a dosage of 0.5 µg. while the somatic antigen of *S. schottmuelleri* was somewhat less active. The typhoid somatic antigen was the least active, requiring about 5 µg. to induce a severe reaction. Typical fever curves obtained in four of the patients following injections of one of the somatic antigens are presented in Figure 1.

Development of tolerance following repeated antigen injections. During the course of consecutive injections of antigen, the response of the pa-

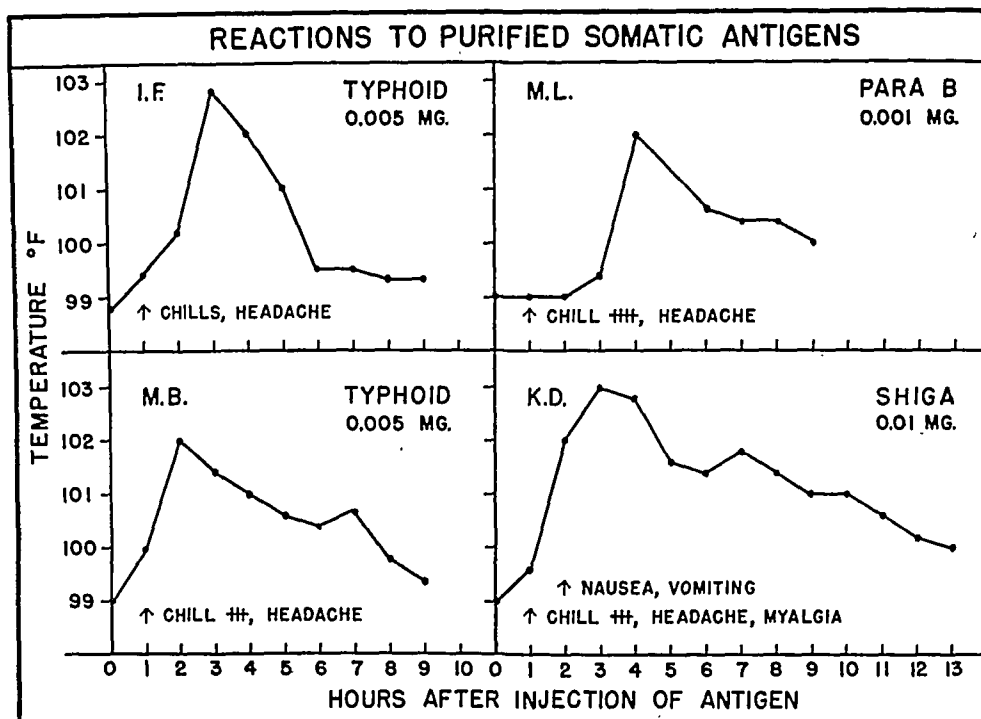


FIG. 1. EFFECTS OF INTRAVENOUS INJECTION OF TOXIC SOMATIC ANTIGENS OF ENTERIC BACILLI IN MAN

tients became less marked until they showed no reaction to its administration. Table I presents results of the effects observed in two of the seven patients studied which are representative. In both

patients, after several consecutive injections, the reactions became less marked until there was no response to the injection of an amount of antigen which had produced symptoms when first given. Further injections increase the degree of this resistance as seen in the results with patient SW.

Specificity of tolerance produced following injection of a toxic somatic antigen. It was of interest to determine whether the resistant state was effective against antigens other than the one with which the tolerance was produced and whether any observed tolerance to the injection of other antigens could be correlated with the presence of circulating antibodies for these materials. The reactions of the patients to injections of heterologous antigens and the "O" agglutinin titers of their sera are summarized in Table II.

From the data presented in Table II it is apparent that tolerance induced by the injection of either Shiga, typhoid or paratyphoid B antigens is effective not only against the toxic effects of the homologous antigen but also against the heterologous antigens. Injections of similar doses of test antigens at the same time in control patients produced characteristic symptoms and febrile re-

TABLE I
Increasing tolerance to injection of toxic somatic antigens

Patient	Day	Antigen* and dose μg.	Fever units	Symptoms†
KD	1	S 0.1	3.6	++
	2	0.2	4.2	+++
	3	0.4	1.5	±
	4	1.0	10.0	++++
	5	1.0	1.2	++
	6	1.0	5.6	++
	7	1.0	0.4	0
	8	1.0	0	0
SW	1	T 1.0	0	0
	2	4.0	1.8	+
	3	8.0	0	0
	4	20.0	0.6	±
	5	100.0	6.6	++++
	6	100.0	2.6	+
	7	100.0	0	0
	8	100.0	0	0

* S = Shiga antigen; T = typhoid antigen.

† Symptoms: +++++, severe; ++++, moderate; ++, mild; +, slight; 0, none.

TABLE II
Heterologous tolerance following injections of
toxic somatic antigens

Pa- tient	Day in- jected	Antigen* and dose	Fever units	Symptoms	"O" agglutinin titer†		
					Typhoid	Para- ty- phoid B	Shiga
KD	1	µg. S 0.1	3.6	++	<8	<8	<8
	4	S 1.0	10.0	++++	<8	<8	<8
	8	S 1.0	0	0	<8	<8	128
	9	T 6.0	1.4	0	<8	<8	128
SW	1	T 1.0	0	0	<8	<8	<8
	2	T 4.0	1.8	+			
	5	T 100.0	6.6	+++			
	10	T 100.0	0	0			
	11	S 5.0	0	0	>2048	2048	<8
	12	B 10.0	0	0	>2048	1024	<8
ML	1	B 0.1	0	0	<8	<8	<8
	2	B 1.0	3.2	++			
	8	B 1.0	0	0			
	9	T 1.0	0	0	64	1024	<8
IF‡	10	S 0.5	1.2	±	64	1024	<8
	1	T 1.0	2.4	++	<8	<8	<8
	2	T 5.0	6.0	+++	<8	<8	<8
	3	S 2.0	10.1	++++	<8	<8	<8
MB‡	1	T 5.0	6.0	+++	<8	<8	<8
	2	B 1.0	2.8	++	<8	<8	<8
	3	S 0.5	4.8	+++	<8	<8	<8

* S = Shiga antigen; T = typhoid antigen; B = paratyphoid B antigen.

† Expressed as the reciprocal.

‡ Controls.

sponses. Patient KD who developed tolerance following eight consecutive doses of Shiga antigen also developed a resistance to the toxic effects of typhoid antigen. At the time this resistance was active against both Shiga and typhoid antigens, the patient's serum showed "O" agglutinins only for *Sh. dysenteriae*. Likewise the tolerance developing in patients SW and ML following the administration of typhoid and paratyphoid B antigens, respectively, was shown to protect against the toxic effects of all three antigens in spite of the fact that serum agglutinins were demonstrable only for the organism which possessed the somatic antigen originally injected or an immunologically related antigen. As would be expected, the sera of patients injected with typhoid or paratyphoid B somatic antigens showed agglutinins for both organisms since these organisms possess a common, somatic, antigenic factor.

These data demonstrate that specific circulating

antibody is not necessary for the action of this tolerance to the toxic action of purified somatic antigens prepared from *S. typhosa*, *S. schottmuel-leri* or *Sh. dysenteriae*.

Duration of the tolerant state. Between four to five weeks after the injections of antigens were stopped, patients KD and ML were again found to be susceptible to the toxic effects of the somatic antigens with which they had been injected as demonstrated by the data in Table III.

TABLE III
Duration of the tolerant state induced by injections
of somatic antigens

Pa- tient	Day* injected	Anti- gen† and dose	Fever units	Symptoms	"O" agglutinin titer†		
					Ty- phoid	Para- typhoid B	Shiga
KD		µg.					
	2	S 0.2	4.2	+++	<8	<8	<8
	4	S 1.0	10.0	++++	<8	<8	<8
	8	S 1.0	0	0	<8	<8	256
ML	40	S 1.0	2.2	+++	<8	<8	256
	3	B 1.0	3.2	+++	<8	<8	<8
	8	B 1.0	0	0	64	1024	<8
	40	B 1.0	2.2	++	<8	512	<8
	114	B 1.0	4.2	+++	<8	256	<8

* Injections were given daily for eight days and subsequently only on the days indicated.

† S = Shiga antigen; B = paratyphoid B antigen.

‡ Expressed as the reciprocal.

These results indicate that individuals have returned to their original state of susceptibility one month after the injections of antigen are stopped. This loss of tolerance occurs in spite of the persistence of high titers of serum antibody for the antigen injected. These observations give additional evidence for the independence of the tolerant state from this antigen-antibody reaction.

DISCUSSION

Tolerance to the toxic action of somatic antigens prepared from *S. typhosa*, *S. schottmuel-leri* and *Sh. dysenteriae* develops readily in man following repeated intravenous injections of these antigens. Symptoms produced following their injection are commonly encountered as part of the clinical manifestations of infection with these bacteria. Furthermore, in animals, the injection of typhoid somatic antigen produced a type of damage to tissues which shows certain similarities of the

lesions observed in fatal cases of typhoid fever in man (5). This may indicate that this potent tissue toxin may play a role in the production of changes observed in the organs of patients dying of typhoid fever.

In the case of typhoid fever, evidence has been presented (6) that the somatic antigen may appear in the free state in the blood stream during the early stages of the disease. It is at least strongly suggestive then that the toxic somatic antigens may have an important role in the causation of some of the clinical and pathological aspects of typhoid and paratyphoid fevers and perhaps also in bacillary dysentery. The phenomenon of tolerance to their toxic action is therefore of considerable clinical interest and may be of importance in the natural infections caused by these organisms. Preliminary tests on a patient convalescent from typhoid fever have indicated that this tolerance may develop during the course of the disease and further studies are now in progress to corroborate this observation (7).

The studies presented here and previous reports (1, 2, 8) indicate that tolerance is not dependent on the presence of specific circulating antibody. The lack of specificity of this resistance with regard to immunological relationships may suggest that some more general function is responsible. Beeson's experiments in rabbits (9) present evidence that this state of resistance is due to an increased activity of the reticulo-endothelial system which removes the pyrogenic materials from the blood stream. So far, no evidence has been presented to show that the tolerance observed in man is due to the same sort of mechanism.

SUMMARY

1. Following repeated intravenous injections of the somatic antigens prepared from the typhoid bacillus, paratyphoid B bacillus and the Shiga dysentery bacillus in man, tolerance develops to the

toxic effects of these antigens as indicated by the failure of the antigens subsequently to produce the characteristic febrile response and constitutional symptoms.

2. Tolerance developing following the administration of any one of these three antigens confers resistance against all three.

3. This resistant state had disappeared in from four to five weeks when the subjects were again tested by injections of these antigens.

4. Tolerance to these somatic antigens does not appear to be related to the presence of circulating antibody for the toxic antigen as measured by the presence of "O" agglutinins.

5. The possible role of this phenomenon in human disease produced by these enteric bacilli is discussed.

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COMPARISON OF THE CONSTANT INFUSION AND URINE COLLECTION TECHNIQUES FOR THE MEASUREMENT OF RENAL FUNCTION¹

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For any exogenous substance which is neither metabolized, stored nor excreted otherwise than in the urine, the rate of excretion must be equal to the rate of infusion under conditions where its plasma level and volume of distribution are constant. This steady state, as indicated by a constant plasma level, will be designated infusion equilibrium.

A simplified method for the measurement of glomerular filtration rate and the effective renal plasma flow based upon the above principle has been described elsewhere (1). In the application of the principle, the rate of infusion, IV (where I is the concentration in mg. per ml. of infusion fluid and V is the volume of fluid injected in ml. per minute), can be substituted for the rate of excretion, UV , in the clearance calculation, i.e., $IV/P = UV/P$. The procedure, therefore, obviates the necessity of bladder catheterization and the errors inherent in urine collection.

The study presented here embodies further observations, obtained under normal and abnormal circumstances, on the correlation of inulin and p-aminohippuric acid clearances as measured by urine collections with those measured simultaneously by the infusion technique, and on the application of the infusion technique to the measurement of p-aminohippuric acid Tm .

EXPERIMENTAL PROCEDURE

Glomerular filtration rate and renal plasma flow were estimated from the clearances (2, 3) of inulin and p-aminohippurate (PAH)² by the usual urine collection technique involving the use of a multi-eyed catheter and

bladder rinses with water and air. The maximum rate of excretion of PAH by the tubules (Tm_{PAH}) was also measured in some of the experiments (3). Three or more serial urine collection periods of 15 to 30 minutes each were measured.

Priming injections of inulin and PAH were given intravenously in amounts calculated to achieve plasma levels of 5 to 20 and 2 to 3 mg. per cent respectively. The approximate volume of distribution for an average normal subject was taken as 15 liters for inulin and 20 liters for PAH. Priming injections of 2 grams of inulin and 0.8 gram sodium p-aminohippurate were frequently used and generally resulted in plasma levels of 10 to 15 and 2.5 to 3.0 mg. per cent respectively. Priming doses were modified for persons of unusual size, and were greatly increased in the presence of edema.

The sustaining infusions were delivered by a pump at a rate calculated, on the basis of estimated renal function, to maintain the desired plasma concentrations. For example, if the estimated filtration rate was 100 ml. per minute and if the plasma inulin concentration desired, and presumably achieved by the priming injection, was 5 mg. per cent then each minute 100×0.05 or 5 mg. would be excreted in the urine. The sustaining infusion, therefore, would deliver 5 mg. of inulin per minute.

For Tm_{PAH} measurements the desired plasma PAH level was roughly calculated for a load/ Tm ratio of 2 from the equation:

$$\frac{\text{Plasma PAH level} \times \text{renal plasma flow (measured or estimated)}}{\text{Anticipated } Tm_{PAH}} = 2$$

The priming PAH dose was calculated from the desired plasma PAH level and a volume of distribution of 20 liters for the individual of average size.

The estimated plasma PAH level was substituted in the formula (3): Anticipated $Tm_{PAH} = UV - 0.83 C_{IN} P_{PAH}$, where C_{IN} represents the anticipated inulin clearance. The equation was solved for the term UV which represents the amount of PAH excreted in the urine per unit of time and, therefore, the amount which must also be delivered by the sustaining infusion.

The infusion pump used in the early experiments consisted of a worm-drive rod powered by a constant speed motor that pushed on the plunger of a 50- or 100-ml. syringe. The rate of delivery with the 50-ml. syringe

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² The authors are indebted to the Medical Research Division of Sharp and Dohme, Inc., of Glenolden, Pa., for generous supplies of sodium-p-aminohippurate in ampules.

was approximately 0.12 ml. per minute, and approximately 0.25 ml. per minute with a 100-ml. syringe. This pump was inconvenient for use in experiments lasting more than three hours since the syringe had to be refilled and the rate of delivery of fluid could be varied only by changing to a larger or smaller syringe.

Subsequently a Bayliss infusion pump was adopted, consisting of a series of rollers which rotated in ferris wheel fashion compressing a short length of the infusion tubing. The pump was powered by a constant speed electric motor. The rate of revolution of the wheel was regulated by a variable speed transmission. Some surging occurred with this pump, but over periods greater than one minute the rate of delivery was constant. Delivery rates with this pump using ordinary intravenous rubber tubing could be varied at will between 1 and 3 ml. per minute.

Generally, infusion equilibrium, as indicated by constant plasma inulin and PAH concentrations, was achieved at the end of one hour in normal subjects. The period allowed for attainment of equilibrium was frequently extended to one and one-half hours, and even then there were a few instances where the plasma levels were still changing during subsequent experimental periods. Throughout this work all infusion technique clearances are based on plasma inulin and PAH levels obtained at the approximate mid-points of the standard urine collection periods.

CHEMICAL METHODS

Inulin was determined by Harrison's modification (4) of the colorimetric method of Alving, Rubin and Miller (5). Protein-free filtrates were obtained by the Somogyi zinc precipitation (6). For inulin analysis, infusion fluids, urines and plasmas were all treated with yeast so that all analyses would be comparable. Plasma, obtained before the injection of inulin, was used as a blank.

PAH was measured by the Bratton-Marshall reaction (7). Since PAH is conjugated by man it is necessary in the infusion technique to determine "total" PAH after hydrolysis. Proteins were precipitated with 3 ml. of 15 per cent trichloroacetic acid per 1 ml. of plasma and 10 ml. of water. Conjugated PAH was hydrolyzed by heating 10 ml. of the filtrate with 1 ml. of 1.1 N hydrochloric acid for one hour in a boiling water bath. One ml. N hydrochloric acid was added to 5 ml. of the hydrolyzed filtrate in a colorimeter tube before addition of the Bratton-Marshall reagents. Plasma blanks were routinely done for each experiment.

A small amount of PAH was destroyed when subjected to the above hydrolysis procedure, the amount increasing with time. On the other hand, hydrolysis of acetyl-PAH increased with duration of heating. The conditions outlined above were chosen as those which permit the greatest degree of hydrolysis with the least destruction of PAH. When known amounts of acetyl-PAH were subjected to the procedure described above, the average recovery was 96 per cent of theory. Since the conjugated compound was apparently not subjected to

destruction during the procedure, it was impossible to hydrolyze the infusion fluids and the plasma filtrates and urines so that they were precisely comparable.

At the high plasma PAH levels maintained during the T_{mPAH} measurements, the fraction of the compound conjugated was so small, and could not be detected, and hydrolysis was, therefore, unnecessary (3).

RESULTS

Glomerular filtration rate and renal plasma flow as measured by the infusion pump technique were compared with simultaneous measurements by the usual methods involving serial urine collections. Unless stated otherwise, all clearance data recorded in the table and figures represent the average of three or more consecutive periods. The clearance values are not corrected for surface area.

For present purposes it was assumed that infusion equilibrium existed whenever there was less than a 6 per cent variation between the serial plasma inulin and PAH concentrations. The variations in plasma level were within the limits of error of the methods of analysis in the majority of experiments.

The average urine/infusion clearance ratio for inulin was 1.00 ($\sigma = 0.054$) in 45 experiments in 26 subjects with normal kidneys and ten with renal disease. These data are summarized in Figure 1.³ The glomerular filtration rates in these subjects ranged from 168 to 14 ml. per minute. In general, there was less variation among the individual clearances in any given experiment as measured by the infusion method than among the clearances as measured by urine collections. For example, comparisons between the two techniques were made during ten consecutive periods in a single experiment. The range in filtration rates measured by urine collection was 122 to 139 ml. per minute, but only 124 to 130 by the infusion technique.

The average urine/infusion clearance ratio for PAH in 26 experiments in 23 of the same subjects was 0.975 ($\sigma = 0.049$). These data are shown in Figure 2.³ It is likely that the slightly low urine/infusion ratio is the result of some loss

³ For the sake of clear presentation, one datum that fell below 25 ml. per minute was omitted from Figure 1, three data that fell below 300 ml. per minute were omitted from Figure 2, and two data that fell below 30 mg. per minute were omitted from Figure 3.

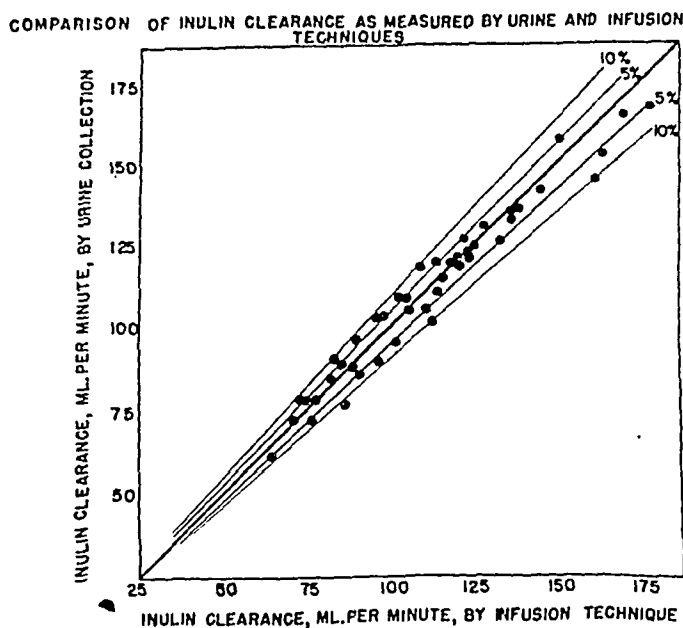


FIG. 1

of PAH during hydrolysis of the urines (see Methods). The filtration fraction (ratio of glomerular filtration to plasma flow) as given by the urine and infusion techniques was calculated for the 26 experiments where both functions were measured simultaneously and the average of the ratio of the two filtration fractions so obtained was 1.045 ($\sigma = 0.055$). The average urine/infusion clearance ratio for inulin in these 26 experiments was 1.02, which in part accounts for the high ratio for the filtration fractions as calculated independently. The relatively low urine PAH clearance due to loss on hydrolysis (see above) is another factor.

During the period throughout which the experiments recorded in the figures were performed, there were also a number of unsuccessful comparisons between the urine collection and infusion techniques. Four instances where there were obvious difficulties with the method of analysis of inulin and PAH are legitimately dismissed from further consideration. There were four other instances where there was either leakage of infusion fluid or infiltration of the fluid into the subcutaneous tissues around the venepuncture. Accidents of this nature invalidate the infusion technique and are attended by rapidly falling plasma inulin and PAH levels.

Urine collections were so inaccurate in four experiments that no comparison of the urine collection and infusion clearances could be made. However, in these four instances, the ratios of the filtra-

tion fractions, as calculated by the two techniques, which are unaffected by inaccuracies of urine collection, were 1.00, 0.95, 0.95 and 0.92.

There were six instances where the plasma inulin levels were not constant enough to assume infusion equilibrium and four instances where the PAH levels were at fault. In all these instances there was a progressive decline in the plasma levels. The infusion clearance usually approached the urine clearance as the experiment progressed, indicating that equilibrium, and thus a valid infusion technique clearance, would have been achieved had the experiments lasted longer.

Attempts to measure filtration rate and plasma flow by the infusion technique were made in five patients with considerable edema. In four instances it was obvious that equilibrium had not been achieved, with progressively falling plasma levels in three instances and very erratic levels in a fourth patient with marked anasarca. In the fifth subject, who had rheumatic heart disease and a pleural effusion but only slight pitting edema of the ankles, the plasma inulin and PAH levels were quite constant for five consecutive 20-minute periods, starting one and one-half hours after the priming infusion. The filtration rate and renal plasma flow were 69.0 and 144 ml. per minute by urine collections and 76 and 144 ml. per minute by the infusion pump technique. However, that there

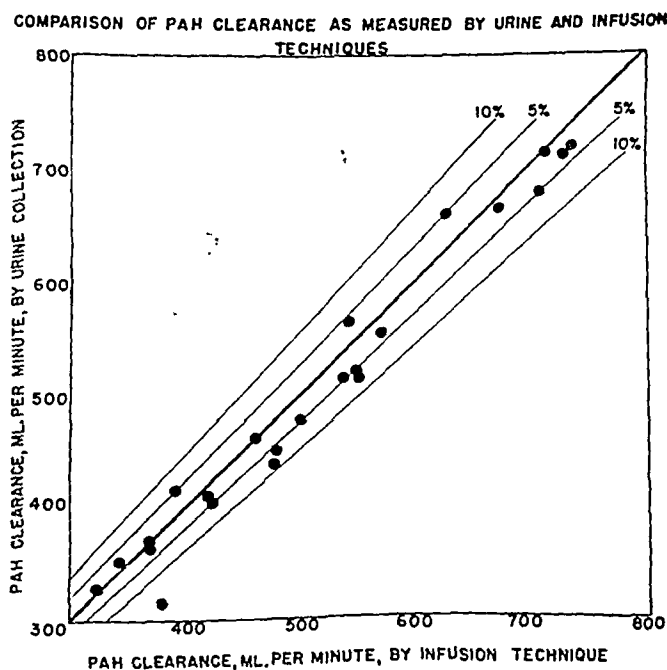


FIG. 2

was not a true equilibrium, in spite of constant plasma drug levels, was indicated by the observation that the concentrations of inulin and PAH in the pleural fluid were respectively 20 and 40 per cent of the concentrations in plasma.

Renal functions as measured by urine collections were much lower than anticipated in three patients with renal disease. The true filtration rates in these patients ranged from 17 to 33 ml. per minute. Amounts of inulin and PAH infused in these experiments exceeded by 40 to 100 per cent the amounts excreted. The calculated infusion technique clearances were, therefore, erroneous, although the plasma levels, obtained at 15- to 20-minute intervals, were quite stable and falsely suggested the presence of equilibrium.⁴ A similar situation in regard to PAH clearances and Tm_{PAH} was observed in the same patients.

The filtration rate is sometimes changed by the intravenous administration of infusions containing large amounts of PAH (8, 9) or mannitol (10). The effect of infusions containing one of these substances, or glucose, on the correspondence between urine collection and infusion technique inulin clearances was studied in 14 experiments. In each experiment the filtration rate was measured during three control periods prior to the administration of the test infusion. At least 30 minutes were per-

⁴ The lower a renal function, the more likely is the possibility of wrongly estimating that function and, therefore, of grossly miscalculating the amount of material to be infused. Generally, the true function is overestimated and too much material is administered in the sustaining infusion, upon which the infusion technique clearance is calculated. In the presence of reduced renal function, even if this function has been overestimated for purposes of calculating the sustaining infusion doses, these doses are relatively small and any amounts administered (in excess of the amounts excreted) are distributed over a large volume of fluid. Change in plasma drug level, therefore, will not be rapid. In addition, the lower the renal function, the greater importance does the initial priming dose assume in determining the plasma level, since relatively small amounts of material are added by the sustaining infusion and little is lost through the kidneys. However, the material infused in excess to that excreted during the three experiments in question should have raised the plasma drug levels by measurable amounts. The observed absence of change in plasma level can only be explained by assuming that material was diffusing into further and further recesses of the extracellular fluid. Why this occurred in these patients with reduced renal function is not known.

mitted to elapse after the beginning of a test infusion before three post-infusion clearances were measured. There were eight instances in which there was a greater than 5 per cent decrease in filtration rate as measured by urine collection, the average decrease being 12 per cent. The average decrease in filtration rate by the infusion technique was 4.1 per cent in the same subjects. In two of these eight subjects there was a slight increase rather than decrease in filtration rate as measured by the infusion technique. In the remaining six subjects there were no significant changes in filtration rates as measured by either technique.

Similar studies were made on the effects of adrenalin, cinchona alkaloids and intravenous triple typhoid vaccine on glomerular filtration rate, renal plasma flow and the filtration fraction. These data are shown in Table I. Adrenalin typically and acutely decreases the plasma flow and increases the filtration fraction (11) while typhoid vaccine results in a gradual but marked increase in plasma flow and a decrease in filtration fraction (11). The effect of the cinchona alkaloids on these functions in man is variable (12), but generally there is a decrease in the plasma flow and an increase in the filtration fraction, especially after quinine. Since the renal functions varied continuously throughout all these experiments, the periods showing the maximum change are recorded in Table I in addition to the averages.

There were seven experiments in which there was an average maximal increase of 35 per cent in the *UV* filtration fraction. The average maximal increase in the *IV* filtration fraction in the same experiments was 20 per cent. The average maximal decrease in the *UV* filtration fraction in five typhoid vaccine experiments and one cinchona alkaloid study was 35 per cent in contrast to an average maximal 27 per cent decrease in *IV* filtration fraction. In all these experiments the *IV* changes in plasma flow were in the same direction as the *UV* changes, and in most the maximal change in the infusion clearances occurred during the same or next period subsequent to the maximum *UV* clearance changes.

Measurements of the maximal rate of excretion of PAH by the renal tubules (Tm_{PAH}) by the *IV* technique were compared to the usual *UV* measurements in 17 experiments. Urine collections

TABLE I
Comparison of renal functions by urine and pump techniques during acute changes

Patient	Procedure	Number of Periods	Inulin Clearance		PAH Clearance		Filtrate fraction		Change in filtrate fraction	
			Calculated on basis of urine collections	Calculated on basis of infusion technique	Calculated on basis of urine collections	Calculated on basis of infusion technique	Calculated on basis of urine collections	Calculated on basis of infusion technique	Calculated on basis of urine collections	Calculated on basis of infusion technique
Las	Control	3	119	114	670	701	<i>per cent</i> 17.8	<i>per cent</i> 16.2	<i>per cent</i>	<i>per cent</i>
	Adrenalin	3	134	125	614	653	21.9	19.2	+23.0	+18.6
	Max. adrenalin	1	136	131	546	624	22.3	19.4	+25.3	+19.8
Har	Control	3	95.6	86.2	400	415	23.8	20.8		
	Adrenalin	3	109	94.6	412	422	26.4	22.3	+10.9	+ 7.2
	Max. adrenalin	1	115	102	390	393	27.3	23.3	+14.7	+12.0
Aik	Control	3	70.9	69.4	412	388	17.2	16.7		
	Adrenalin	3	63.8	67.8	289	324	22.1	20.9	+28.5	+25.2
	Max. adrenalin	1			263	298	25.0	22.1	+45.5	+32.4
Hen	Control	3	152	164	654	661	23.2	24.7		
	Quinine	6	146	156	502	584	29.0	26.7	+25.0	+ 8.1
	Max. quinine	1	136	147	368	518	36.9	29.3	+59.0	+18.6
Mil	Control	3	115	116	624	608	18.4	19.1		
	Quinine	6	110	113	417	499	26.4	22.6	+43.5	+18.4
	Max. quinine	1	111	111	378	455	29.4	24.4	+59.0	+27.8
Joh	Control	3	103	95.4	509	528	20.3	18.1		
	Quinine	5	95.1	88.5	419	455	22.7	19.5	+11.8	+ 7.7
	Max. quinine	1	86.2	84.2	374	499	25.2	20.6	+24.1	+13.8
Gri	Control	2	88.6	86.5	447	475	19.8	18.2		
	Cinchonine	6	86.0	86.1	422	439	20.4	19.7	+ 3.0	+ 8.2
	Max. cinchonine	1	88.9	87.6	380	415	23.4	21.1	+18.2	+15.9
								Average	+20.7	+13.3
								Maximum	+35.1	+20.0
Mil	Control	3	123	121	512	541	24.0	22.3		
	Quinidine	6	125	128	616	561	20.3	22.8	-15.4	- 2.2
	Max. quinoline	1	116	123	710	678	16.4	18.2	-31.6	-18.4
Sol	1st typhoid		146	131	671	612	21.8	21.4		
	Typhoid	6	135	136	830	820	16.3	16.6	-25.5	-22.5
	Max. Typhoid	1	136	141	999	978	13.6	14.4	-37.6	-32.8
Per	1st typhoid	1	79.9	72.7	361	304	20.5	23.9		
	Typhoid	5	77.5	73.4	510	426	15.2	17.2	-25.9	-28.1
	Max. typhoid	1	71.9	72.4	557	503	12.9	14.4	-37.0	-39.9
Rio	1st typhoid	1	74.9	79.2	483	453	14.7	15.3		
	Typhoid	5	71.4	76.1	542	532	13.2	14.3	-10.2	- 6.5
	Max. typhoid	1	66.2	74.3	593	570	11.2	13.5	-23.9	-11.8
Aik	1st typhoid	1	68.6	66.8	354	348	19.4	19.2		
	Typhoid	6	60.6	64.8	414	395	14.6	16.4	- 4.8	-14.6
	Max. typhoid	1	53.6	64.1	506	479	10.6	13.4	-45.5	-30.2
								Average	-16.4	-14.8
								Maximum	-35.1	-26.6

were obviously faulty in one experiment, while in two the load of PAH was below that necessary to saturate the excretory mechanism. In another four experiments the load of PAH was apparently too large (load/ Tm_{PAH} ratios greater than 4.0)

to permit the attainment of equilibrium. Eight of the remaining ten experiments (load/ Tm_{PAH} ratios all between 1.5 and 4.0) are summarized in Figure 3.³ The calculations for the Tm_{PAH} values shown in Figure 3 involve the respective UV and

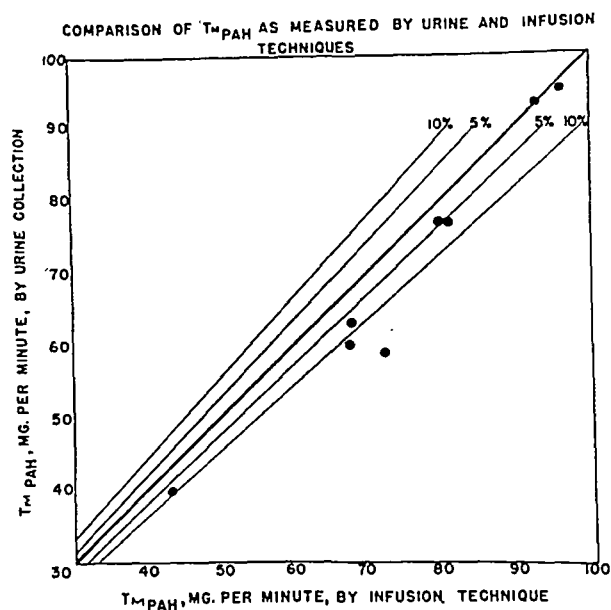


FIG. 3

IV filtration rates, which in themselves are subject to some differences, especially during the infusion of the relatively large amounts of PAH necessary for the Tm_{PAH} measurements (see above). The actual mgs. per minute of PAH infused and excreted afford a direct comparison between the *UV* and *IV* techniques. The average ratio between PAH infused and excreted was 0.95 in the ten experiments with a range of 0.84 to 1.00 while the average ratio of *UV* to *IV* Tm_{PAH} was 0.93.

COMMENTS

Clearances of inulin and PAH when calculated on the basis of the constant infusion technique compare favorably with the values obtained by the usual urine collection techniques in a series of patients with both normal and moderately reduced renal functions. Attention to a number of details is essential.

It should be stressed that great care must be taken in the interpretation of the *IV* clearances in patients with reduced kidney function. In three such instances where the sustaining infusions had been calculated for clearances that had been overestimated, the *IV* clearances were considerably higher than the true values, in spite of constant plasma drug levels.⁴ When the infusions were based on more accurately estimated clearances good correspondence between the two techniques was

obtained, even though the filtration rate was very low. Unfortunately, the existence of apparently stable plasma inulin and PAH levels during the three experiments discussed above makes it impossible to be certain, without simultaneous urine collections, that the infusion technique clearances represent true values when renal functions are less than 60 per cent of normal.

In contrast to the situation described above, lack of equilibrium in the presence of edema was readily indicated by changing plasma drug levels. In the presence of any significant amount of edema, equilibrium is difficult to achieve. It is felt, however, that successful *IV* clearances could be obtained under these circumstances if the proper relations between the priming and sustaining infusions and the excretory rate could be achieved, and if a considerably prolonged period for equilibrium were allowed.

Under a variety of circumstances where inulin or PAH clearances were acutely altered, the infusion technique values showed the same directional changes as were indicated by the *UV* clearances, while the magnitudes of the *IV* clearance changes were usually less. Although equilibrium is impossible under continuously changing conditions such as were present in the experiments recorded in Table I, the infusion technique clearances reflected the actual changes with sufficient rapidity and at times with sufficient accuracy to suggest that the virtual volume of distribution of inulin and PAH concerned in the acute readjustments are considerably smaller than the true volumes of distribution as attained at equilibrium. Landowne and Alving (13) have commented that a prolonged period of time would be required for a given change in renal function to be reflected by significant changes in plasma drug concentrations. Their calculations, however, were based on the assumption that the redistribution of the compounds would immediately involve all extracellular fluid.

In addition to the measurement of glomerular filtration rate and renal plasma flow by the infusion technique it is possible to measure Tm_{PAH} . When the load of PAH (see Methods) to Tm_{PAH} ratio is kept between 2 and 4, reasonably good checks between the infusion and urine collection techniques were obtained.

SUMMARY

1. Good correlations between the infusion and urine collection techniques for measuring glomerular filtration rate and effective renal plasma flow were obtained in 45 experiments with inulin and 26 experiments with PAH in subjects with normal or moderately reduced renal function, and who did not have more than minimal edema.

2. The infusion technique may be applied to the measurement of the maximal rate of excretion of PAH by the renal tubules.

3. Unsuccessful attempts to measure renal function by the infusion technique resulted from failure to attain equilibrium between the rates of infusion and excretion, especially in the presence of edema.

4. The infusion technique clearance may be erroneous in short experiments in subjects whose clearances are less than 60 per cent of normal. It is not possible in this circumstance to detect the erroneous experiments without concomitant urine collection clearances.

5. The infusion technique clearances reflect acute changes in filtration rate and renal plasma flow, but are usually damped in magnitude.

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EVALUATION OF NEUROGENIC AND HUMORAL FACTORS IN BLOOD PRESSURE MAINTENANCE IN NORMAL AND TOXEMIC PREGNANCY USING TETRAETHYLAMMONIUM CHLORIDE

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Elevation of the arterial blood pressure is one of the most characteristic signs of toxemia of pregnancy. That this blood pressure elevation must reflect an increase in peripheral resistance in the toxemic patient is well recognized. More and more emphasis has been placed on the observation of vasoconstrictor phenomena in this illness so that arteriolar spasm is now thought to represent a frequent if not universal concomitant of toxemia. Since, however, it is well established that constrictor responses may be initiated through both neurogenic and humoral mechanisms, the question as to the primacy of either of these factors in the hypertension of toxemia remains unanswered.

Designation of the specific hypertensive disease of pregnancy as *toxemia* implies a primary humoral disorder. Despite the lack of conclusive evidence to support this concept, it has gained wide acceptance. Investigators have been occupied with the search for hormones, pressor agents and enzyme inhibitors peculiar to gestation. Although significant discoveries have been made, none has clarified the nature of blood pressure control in either toxemia or normal pregnancy.

The recent development of pharmacologic agents which induce transient paralysis of the autonomic nervous system has provided a valuable adjunct for study of the mechanisms involved in blood pressure control. Tetraethylammonium chloride (TEAC), by blocking sympathetic impulse transmission at the ganglionic level, abolishes neurogenic tone. Failure to obtain a fall in blood pressure following TEAC suggests the activity of humoral agents. After sympathetic block, the blood pressure remains responsive to pressor substances and thus must be maintained by humoral mechanisms together with intrinsic vascular tone. Accordingly, an assay of TEAC responses in normal and toxemic pregnancy was undertaken in an

effort to throw light on the relative importance of humoral and neurogenic factors in blood pressure maintenance in these conditions.

MATERIAL AND METHODS

Forty-three patients were studied, including the following groups: normal non-pregnant controls, 10 subjects; normal term pregnancy, 10 patients; and toxemia of pregnancy, 23 patients. In the toxemic group, 12 patients were designated as having preeclampsia, five eclampsia, and six had toxemia superimposed on an antecedent hypertension. The criteria for classification of the above patients are given at the end of this section.

TEAC¹ studies were performed on all pregnant patients in both the prepartum and postpartum periods. Prepartum responses of normal pregnancies were recorded as near term as possible. Following delivery the same patients were tested again, in every case between 24 and 48 hours postpartum.

In the toxemia group, antepartum TEAC responses were recorded at the time when clinical signs and symptoms of toxemia were most prominent and prior to the institution of therapy. After parturition, TEAC responses were observed on one or more occasions between the second and seventh postpartum day. In all cases it was attempted to secure responses coincident with the recovery from toxemia as determined by clinical and laboratory evidence. In addition, 12 patients were tested repeatedly during the ninth month of pregnancy and the puerperal period at times when the clinical course suggested either abatement or increased severity of the toxemic state. Responses were also recorded on these patients at intervals up to six months postpartum for control purposes.

Normal non-pregnant women, utilized in this study as a control group, were subjected to a single test of blood pressure response following TEAC.

All studies were conducted with the patient in the supine position. After a control period during which the blood pressure was allowed to stabilize, 4 cc. of TEAC (400 mg.) were injected intravenously to accomplish autonomic blockade. Following the injection the blood pressure was recorded (sphygmomanometer) at half-minute intervals for six minutes and at one-minute intervals for 10 to 30 minutes thereafter. In this

¹ Etamon chloride supplied by Parke, Davis and Company, through the courtesy of Dr. E. C. Vonder Heide.

study the *control blood pressure* represents the mean of five or more readings following stabilization of the pressure in the recumbent position. The lowest point to which the pressure descended in the first five minutes following injection has been termed the *TEAC floor*.

Criteria for the diagnostic classification of the various groups studied were as follows:

Normal non-pregnant controls: These included healthy nulliparous females from the hospital staff and medical students. Their ages ranged from 22 to 37 years. None gave historical or present evidence of hypertension.

Normal term pregnancy: Included in this group were four primiparae and six multiparae who had been under observation in the prenatal clinic. Their ages ranged from 16 to 36 years. None had evidence of present or past hypertension, or previous toxemia.

Toxemias of pregnancy: Hypertension, albuminuria and edema occurring in the third trimester were used as the prime differential diagnostic points of this group. Subjective symptoms (headache, epigastric pain, scotomata) were given only secondary consideration. The ages of the 23 toxemic patients ranged from 14 to 42 years, with a mean of 25 years.

Twelve of the 23 patients in this group were considered to be *preeclamptic*. Nine were primiparae and three multiparae. From the standpoint of clinical severity, three patients had Grade I and nine patients Grade II preeclampsia. Clinical and laboratory examinations during hospitalization together with observations for four to six months postpartum served to confirm the diagnosis. The majority of patients, however, showed albuminuria and hypertension of such degree as to leave no question as to clarity of the diagnosis at the time of confinement.

Convulsions were observed in five patients (three primiparae, two multiparae) and these have been classified as *eclampsia*. The seizures occurred postpartum in two patients who had been considered preeclamptic prior to delivery.

In six patients who presented the classical toxemia triad there was adequate evidence of *pre-pregnant hypertension*. Of these, two were primiparae and four multiparae. Previous blood pressure observations and urinalyses were available from hospital records to indicate a further elevation of the hypertension and the appearance of albuminuria with relation to the current pregnancy. In addition, a three to six month follow-up of these patients following delivery has provided further evidence of the accuracy of the diagnosis at the time of hospitalization.

RESULTS

The TEAC blood pressure response and floor deviate strikingly and consistently from the normal, both in toxemia of pregnancy and in normal pregnancy at term. In toxemia, the floor is consistently elevated and falls to normal after delivery and recovery from toxemia. In normal term pregnancy, the floor is consistently depressed and rises to normal following parturition.

Normal non-pregnant controls: The results are indicated in Figure 1. TEAC induces very little fall in blood pressure. The mean control blood pressure for the group was 112/72 mm. Hg, and the mean TEAC floor was 103/67 mm. Hg. The

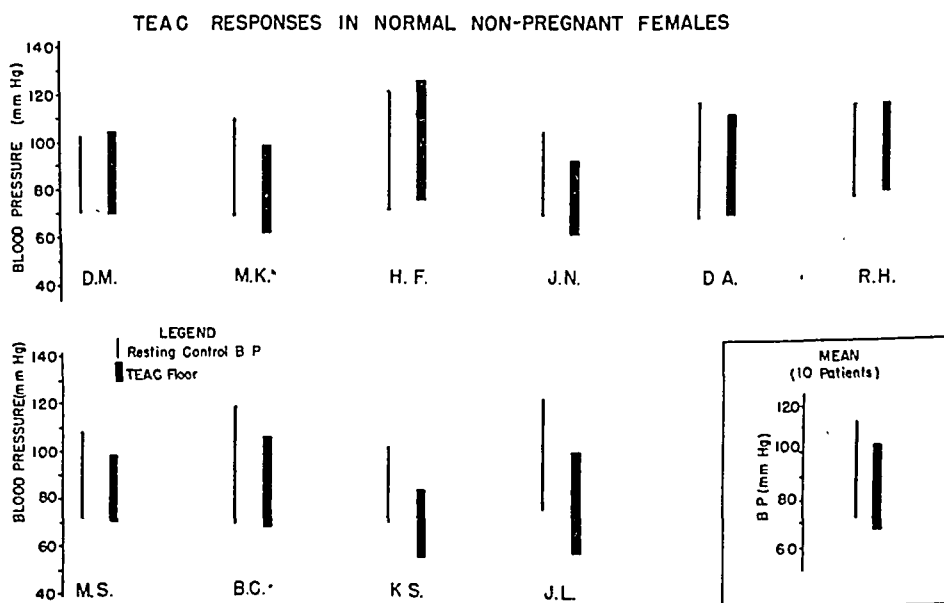


FIG. 1. BLOOD PRESSURE RESPONSES OF NORMAL NON-PREGNANT FEMALES FOLLOWING AUTONOMIC BLOCKADE WITH TEAC

The thin vertical line represents the control blood pressure and the heavier line the TEAC floor. At the lower right is depicted the mean group response. Note the meager magnitude of the response (10/5 mm. Hg).

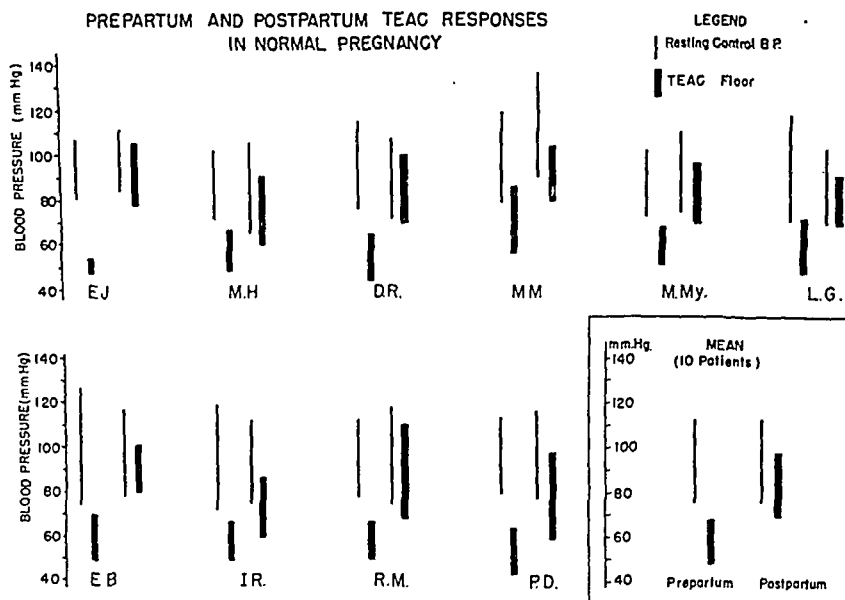


FIG. 2. PREPARTUM AND POSTPARTUM TEAC BLOOD PRESSURE RESPONSES AND FLOORS IN NORMAL PREGNANCY

The responses are grouped in pairs, the first of each pair representing the prepartum response at term and the second the response within 48 hours following delivery. Mean prepartum and postpartum responses for the entire group are shown at the lower right. Note the exceedingly low TEAC floors obtained prepartum and the striking rise to "normal" levels immediately after parturition.

magnitude of these responses is similar to those reported by Lyons in normotensive men and women subjects (1) and to our own observations of young normotensive men (2).

Normal term pregnancy: The results are shown in Figure 2. In each of 10 patients the TEAC floor was strikingly low, ranging from 54/44 to 86/56 mm. Hg, with a mean of 68/49 mm. Hg. This finding is in striking contrast to the TEAC floors in non-pregnant normotensive females (Figure 1) which ranged from 84/56 to 126/80 mm. Hg, the mean being 103/67 mm. Hg, although the control blood pressures in both groups were similar. After delivery, the TEAC floor rose to "normal" levels in all patients.

Toxemias of pregnancy: In all 23 cases of toxemia, whether preeclamptic, eclamptic or pre-pregnant hypertension with superimposed toxemia, the TEAC floor was markedly higher than that found in the group of normal term pregnancy, and fell within the normal range of values upon recovery from toxemia, following delivery or occurring spontaneously. A comparison of the prepar-

tum and postpartum TEAC blood pressure floors in toxemia and normal term pregnancy is shown in Figure 3. It is notable that in no instance of toxemia did the diastolic TEAC floor reach levels below 80 mm. Hg, while in normal term pregnancy it did not exceed levels of 56 mm. Hg.

Preeclampsia: The prepartum and postpartum (recovery period) blood pressures and TEAC floors of the 12 patients in this group are shown in Figure 4. During the *toxemic phase*, there is a moderate though variable blood pressure response to TEAC, the mean control pressure being 159/107 mm. Hg, and the mean TEAC floor 131/94 mm. Hg. The floor is higher than that of normal non-pregnant females (mean 103/67 mm. Hg) and differs strikingly from that of normal term pregnancy (mean 68/49 mm. Hg). After delivery and with clinical recovery from the toxemia, the TEAC floor falls consistently to normal levels, the mean floor being 97/67 mm. Hg. Comparison of the latter figure to the mean postpartum floor of normal term pregnancy (98/70 mm. Hg) and to the mean floor for normal non-pregnant females

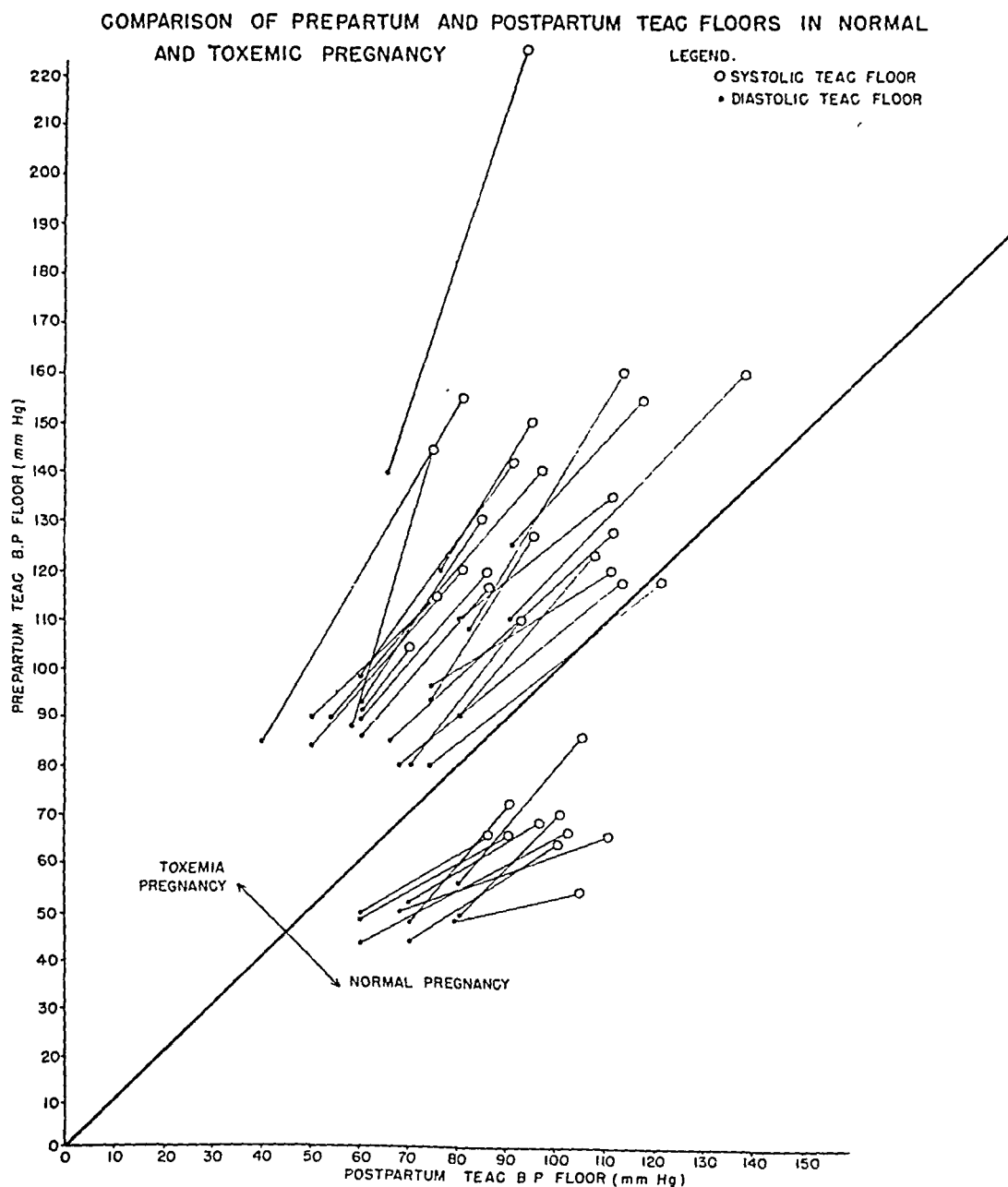


FIG. 3. COMPARISON OF PREPARTUM AND POSTPARTUM TEAC BLOOD PRESSURE FLOORS IN NORMAL AND TOXEMIC PREGNANCY

Systolic and diastolic TEAC blood pressure floors have been plotted against each other in this graph, prepartum studies along the ordinate and postpartum studies along the abscissa. The circles represent systolic floors and the dots diastolic floors. It should be pointed out that if prepartum and postpartum floors were the same, the points plotted would fall on the diagonal. It is obvious that on the basis of blood pressure assay with TEAC all pregnant patients studied fall into two distinct groups, the 23 toxemias above and the 10 normal pregnancies below the diagonal. It can be seen that in those patients above and to the left of the diagonal the postpartum TEAC floor has fallen (toxemias), whereas in those below and to the right of the diagonal, the floor has risen postpartum (normal pregnancy). Note that in toxemia the prepartum diastolic TEAC floor did not descend below 80 mm. Hg, while in normal term pregnancy, the highest prepartum diastolic floor was only 56 mm. Hg. The similarity of systolic and diastolic floors in both groups postpartum should be noted.

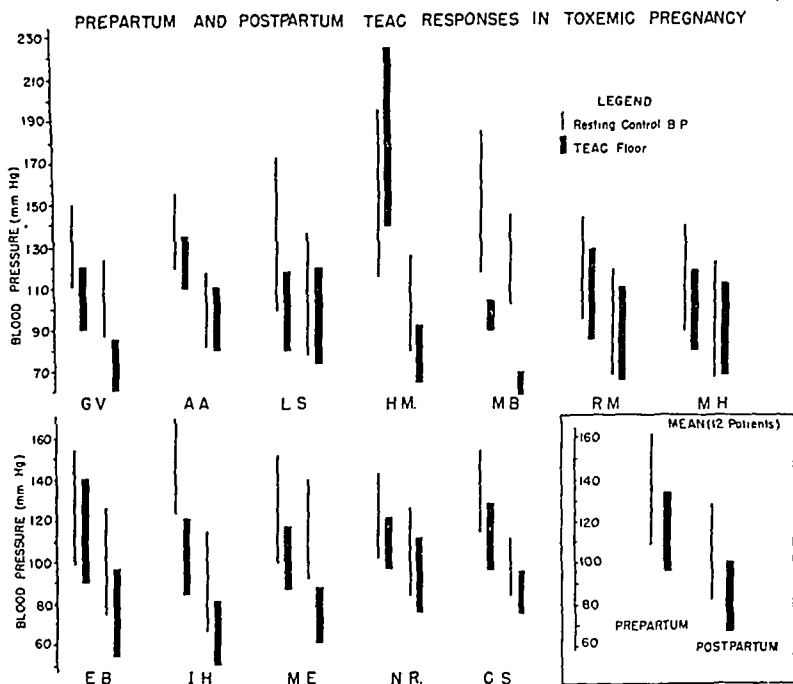


FIG. 4. PREPARTUM AND POSTPARTUM TEAC BLOOD PRESSURE RESPONSES AND FLOORS IN PREECLAMPSIA

As in Figure 2, the responses are paired. The first of each pair represents the TEAC response at the height of the toxemia, and the second shows the response coinciding with postpartum recovery from toxemia (between the second and seventh postpartum day in these patients). Rapid descent of the postpartum TEAC floors from abnormally high prepartum (toxemic) test levels is apparent.

(103/67 mm. Hg) points up the close similarity of these data.

Eclampsia: The results are shown in Figure 5. The TEAC responses and floors in the five cases studied are qualitatively similar to those observed in preeclampsia, but are more striking in that the TEAC floors are higher (mean 135/103 mm. Hg). Fall in the floor to the normal range after recovery is more dramatic for this reason (mean 95/71 mm. Hg).

Essential hypertension with superimposed toxemia: The results are shown in Figure 5. They are similar to those seen in the two groups above. Both the prepartum and postpartum control blood pressures are higher than in the other toxemic groups, and the prepartum TEAC floor is somewhat higher (mean 145/95 mm. Hg), although the postpartum TEAC floor is essentially the same (mean 97/67 mm. Hg).

The mean TEAC blood pressure responses and floors are compared graphically in Figure 6. For

this purpose all patients with toxemia have been considered as a single group, whether preeclamptic, eclamptic or prepregnant hypertension with superimposed toxemia. Prepartum and postpartum studies are compared with those of normal pregnancy and with responses of non-pregnant controls. The mean prepartum TEAC floor of all toxemias is 135/96 mm. Hg, and descends to 97/67 mm. Hg with postpartum recovery.

Changes in the TEAC floor and blood pressure response during fluctuations in the severity of toxemia

Figure 7 illustrates the changing responses and floors in four patients whose clinical courses showed fairly marked fluctuation in both the antepartum and puerperal periods. The TEAC floor consistently was observed to rise and fall in relation to exacerbations and remissions of the toxemic state. These fluctuations in TEAC floor were re-

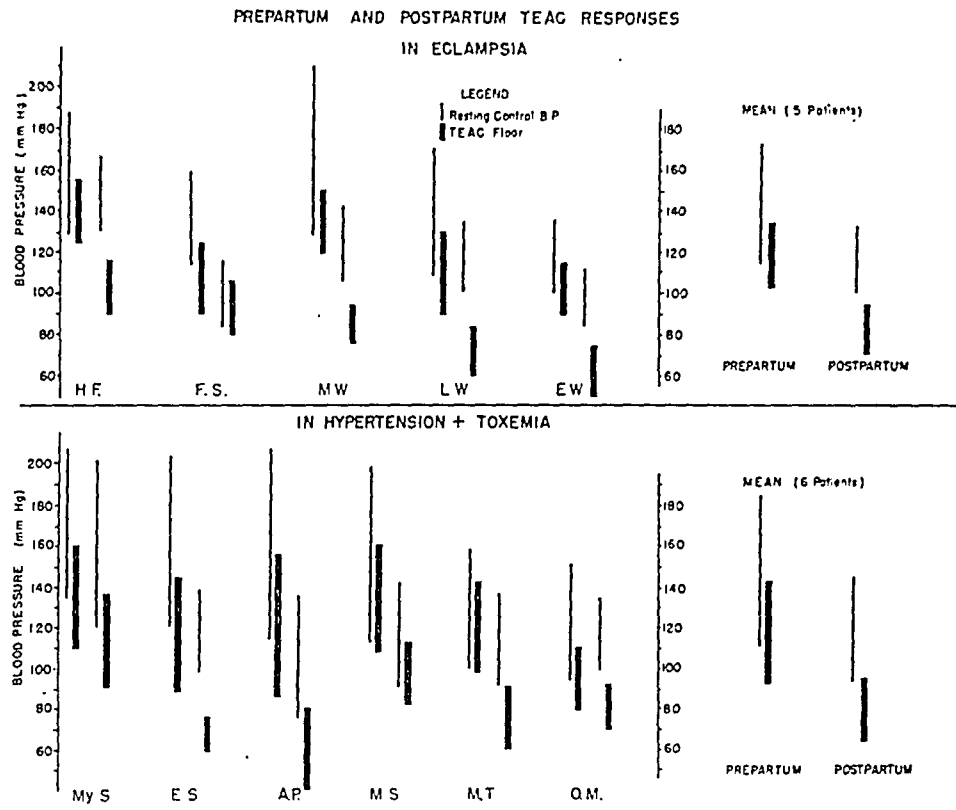


FIG. 5. PREPARTUM AND POSTPARTUM TEAC BLOOD PRESSURE RESPONSES AND FLOORS IN ECLAMPSIA AND HYPERTENSION WITH SUPERIMPOSED TOXEMIA

As in Figure 4, the second of the paired responses was obtained postpartum during the phase of recovery from toxemia. As in preeclampsia, the TEAC floors are abnormally elevated at the height of the toxemia and descend rapidly to "normal" levels during the recovery period from toxemia following delivery.

peatedly noted to be independent of the height of the control blood pressure.

DISCUSSION

Acheson and Moe (3) in animals and Lyons, *et al.* (4) in man have demonstrated that TEAC produces a transient blockade of the autonomic nervous system at the ganglionic level, and that the resulting fall in blood pressure is due primarily to a decrease in peripheral resistance (arteriolar tonus). In addition it has been shown that pressor agents (angiotonin, epinephrine) which act directly on the blood vessels are capable of producing pressor responses when autonomic impulse transmission has been completely eliminated by TEAC (5).

Evidence for the completeness of the sympathetic blockade induced by acute intravenous doses of 400 mg. of TEAC is presented in detail elsewhere (6). A brief summary of the pertinent information is appropriate here: (a) the TEAC floor

parallels but is consistently lower than the blood pressure floor induced by high spinal anesthesia to T-3 levels; (b) the TEAC floor is not lowered further by doubling the dose in the same individuals; and (c) TEAC abolishes the minute cold pressor blood pressure effect, a response mediated through the sympathetic nervous system (7). Thus, it may be concluded that the blockade achieved is sufficiently complete for the purposes of this investigation, since a constant dose was employed in all groups studied and in the same individuals under the varying conditions of study.

That the sympathetic nervous system mediates active vasomotor control of the arteriolar bed is now well established. In this clinical assay of responses following autonomic blockade with TEAC, the active neurogenic component of blood pressure maintenance may be assumed to have been eliminated, and the resulting blood pressure (floor) is therefore maintained by humoral and intrinsic mechanisms.

COMPARISON OF MEANS.

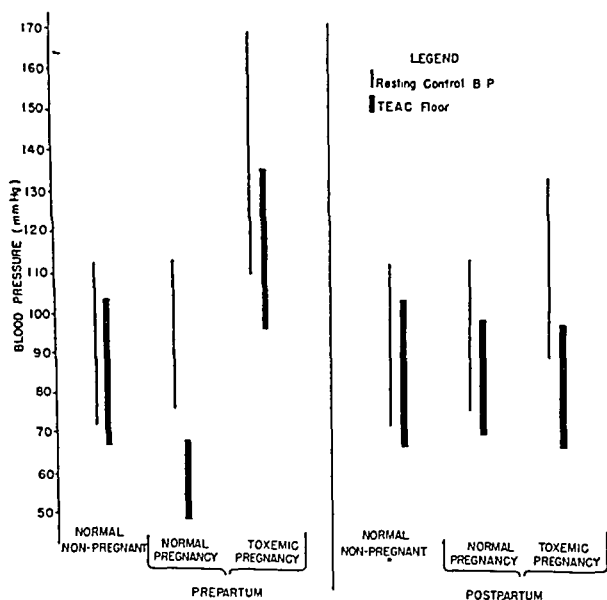


FIG. 6. MEAN PREPARTUM AND POSTPARTUM TEAC BLOOD PRESSURE RESPONSES OF NORMAL TERM PREGNANCY AND COMBINED TOXEMIA GROUPS

For comparison purposes the mean response of normal non-pregnant controls has been included here. The toxemia mean includes all 23 cases studied, whether pre-eclamptic, eclamptic or pre-pregnant hypertension with toxemia. Postpartum shift of the TEAC floors of the pregnant groups to levels exactly corresponding to that of the non-pregnant controls should be noted.

It is obvious from Figure 1 that normal non-pregnant women are, generally speaking, poor responders to TEAC. Despite slight variations from individual to individual, the mean blood pressure fall of 10/5 mm. Hg reflects the minor role played by sympathetic vasoconstrictor tone in maintaining blood pressure in non-pregnant normals.

Rapid shifting of postpartum TEAC floors (rising floors in normal pregnancy, falling floors in toxemia) suggests a striking difference in the mechanism of blood pressure maintenance at term of normal pregnancy from that involved in sustaining the hypertension of toxemia. Blood pressures in prepartum term normals invariably fell to exceedingly low levels with sympathetic blockade, indicating neurogenic domination in this group. Release of sympathetic tone with TEAC in toxemia caused varying degrees of blood pressure fall, but diastolic TEAC floors rarely descended below 90 mm. Hg, suggesting activity of humoral

mechanisms in sustaining hypertension in these patients.

The fall in blood pressure induced by TEAC in man (1, 8) is not associated with relevant changes in cardiac output or blood volume. However, term pregnancy is attended by blood volume alterations and by venous pooling in the lower extremities due to the increased venous pressure in the tributaries to the inferior vena cava which results from the pressure of the gravid uterus. Therefore, consideration must be given these changes as possible factors influencing the shifts observed in the TEAC response and floor in the pregnant patients studied. In toxemia the plasma volume is either normal or decreased (9), whereas it is regularly increased in normal term pregnancy (10). Since the levels of both the control blood pressures and TEAC floors are diametrically opposite to what might be expected from the blood volume shifts after delivery, this possibility is eliminated as a cause for the divergent floors.

Likewise, since the factors which increase the venous pressure in the lower extremities are essentially the same in toxemia and normal term pregnancy, it is probable that the venous pressure is similarly elevated in both conditions, returning to normal after delivery. Therefore, the opposite effects of TEAC in normal and toxemic pregnancy cannot be due primarily to differences in venous pressure.

Since TEAC causes transient postural hypotension, perhaps through lessening neurogenic venous tone, it is quite possible that under conditions of term pregnancy, when the venous pressure in the lower extremities is elevated, release of venous tone may exaggerate venous pooling and, therefore, contribute to the striking fall in blood pressure which occurs in normal term pregnancy. Regardless of the relative roles of arteriolar and venous tone in the blood pressure response to TEAC in normal term pregnancy, it is most probable that factors other than neurogenic tone, namely, humoral factors, contribute to the elevated TEAC blood pressure floor in toxemia. The relative roles of venous pooling and arteriolar tone in the blood pressure response to TEAC in pregnancy are being further investigated.

The contention that the extent of the decrease in pressure is largely dependent on the initial elevation of pressure is untenable here. Many of the

TEAC RESPONSES IN TOXEMIC PREGNANCY

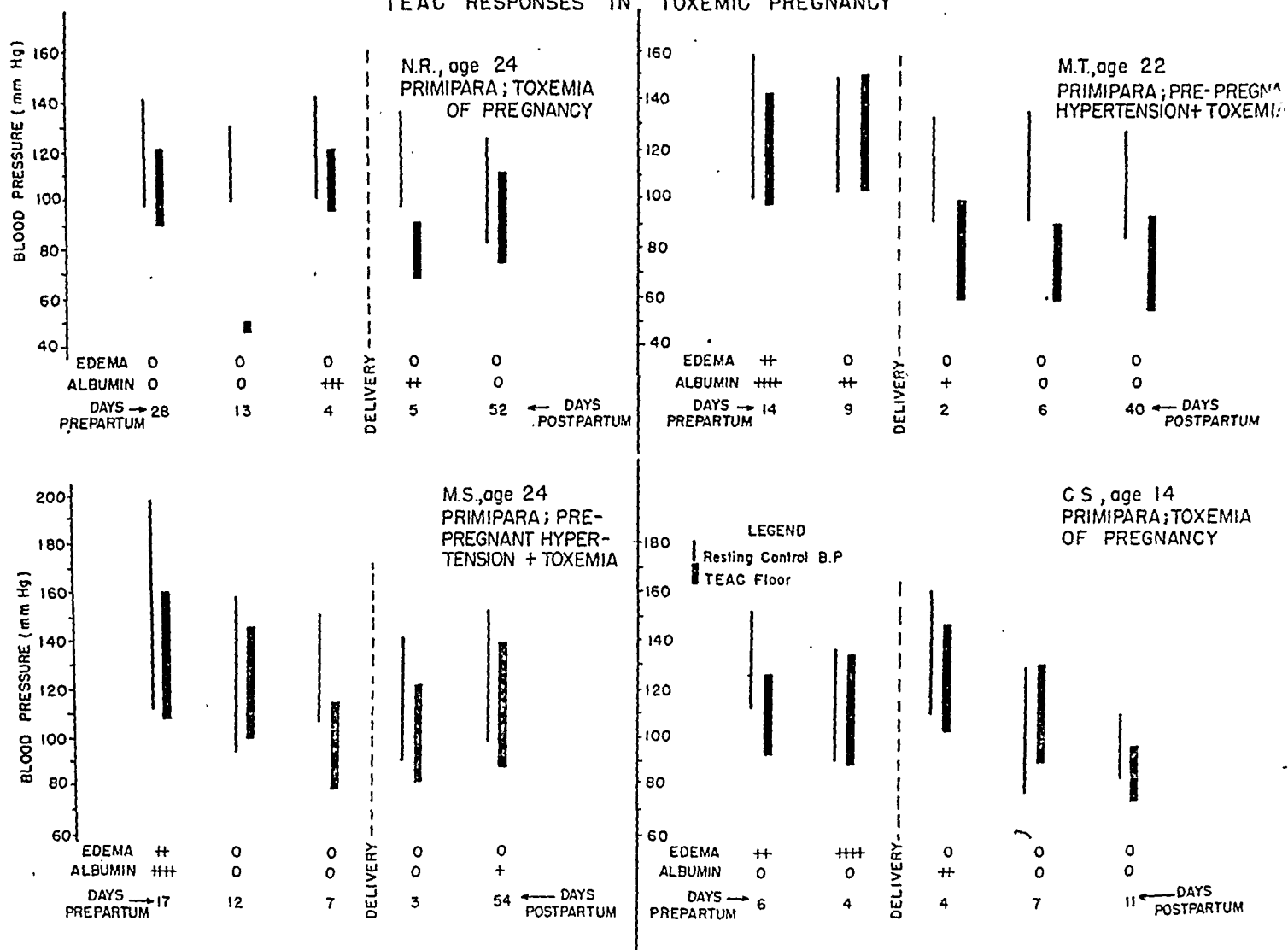


FIG. 7. CHANGES IN THE TEAC BLOOD PRESSURE RESPONSE AND FLOOR DURING FLUCTUATIONS IN THE SEVERITY OF TOXEMIA

Pictured are the responses of four representative toxemia patients who were tested repeatedly during prepartum and postpartum periods in an effort to correlate TEAC responses and floors with fluctuations in the severity of toxemia.

Patient N. R. was considered toxemic 28 days prepartum, because of progressive rise in blood pressure to persistent hypertensive levels observed in the course of prenatal visits. Following hospitalization, bedrest and restricted salt diet, the blood pressure returned to normal and the patient was discharged. Thirteen days prepartum she was totally free of signs and symptoms of toxemia and a TEAC response typical of normal term pregnancy was obtained. Hypertension and albuminuria appeared one week prepartum and on the fourth day prior to delivery the TEAC response and floor conformed to the toxemic pattern.

Patient M. T. illustrates a case in which the toxemic symptomatology and toxemic blood pressure responses to TEAC persisted until delivery.

Patient M. S., a prepregnant hypertensive with superimposed toxemia, showed marked prepartum remission of toxemia. The TEAC blood pressure floors likewise showed improvement (descended) simultaneous with the clinical improvement although responses similar to those of normal term pregnancy were not obtained.

Patient C. S. exhibited severe edema and moderate hypertension in the antepartum period. TEAC floors were consistently elevated. Marked exacerbation of hypertension and appearance of albuminuria followed delivery, and the TEAC floor rose still higher. With recovery from toxemia, the TEAC floors gradually returned to normal.

eclampsics and pre-pregnant hypertensives persisted with elevated blood pressure following the disappearance of other toxic signs. Despite this, the TEAC floors invariably descended during the puerperium. The results in normal pregnancy further refute this concept by consistently showing rising TEAC floors immediately postpartum even though initial pressures were identical with prepartum levels.

Thus, the data presented offer physiologic support for the humoral theory of pregnancy toxemia so far as the hypertension is concerned. Simultaneously, attention is directed to the nature of alteration of blood pressure control in normal pregnancy. The evidence suggests that the blood pressure of the normal non-pregnant female is supported largely by humoral (plus intrinsic) tone. Although the pressure remains normal or low during normal pregnancy, at some time during gestation the humoral component is relegated to a minor role, and neurogenic (plus intrinsic) tone predominates at term. Immediately postpartum, humoral factors again come into play presumably to the same degree as in control normotensive subjects. When toxemia develops in the course of pregnancy, humoral mechanisms support a considerable portion of the associated blood pressure elevation. Nevertheless, toxemic blood pressures are frequently reduced following sympathetic blockade, indicating that neurogenic mechanisms are still operative. Whether the blood pressure response to TEAC is due to release of some remaining neurogenic arteriolar or venous tone or to a combination of the two, remains to be determined.

Research in pregnancy toxemia recently has centered about the search for increased quantities of humoral agents and deficiencies of inhibitor substances. Page has found plasma angiotonase levels elevated four to ten times in normal pregnancy (11). This finding is of increasing interest in view of the evidence for diminished humoral tone demonstrated in normal term pregnancies in the data just presented. Histaminase and pitocinase are likewise elevated in normal pregnancy. Deficiencies of progesterone and estrogens and increased amounts of chorionic gonadotropin have been described in toxemia. Interestingly, the temporal relationships of rising and falling titers reported for many of these substances (12) corre-

spond closely to the rising and falling of the TEAC floors before and after delivery.

There is considerable evidence that clinical assay with TEAC may offer valuable assistance in the diagnosis of toxemia and in following the course and severity of the disorder. Figure 7 shows how, in general, the height of the TEAC floor parallels the severity of the toxemia. The highest diastolic floors observed occurred in two patients with eclampsia and in the most severe case of preeclampsia studied. Cases which fluctuated in severity showed corresponding fluctuation in the height of the TEAC floor.

From the standpoint of diagnosis there is as yet no evidence that TEAC assay can differentiate between toxemia and essential hypertension uninfluenced by pregnancy. The greatest diagnostic assistance offered is in those pregnant patients admitted with borderline elevations of blood pressure. On the basis of the current study, it may be stated that a patient who experiences a TEAC response resulting in a diastolic blood pressure floor considerably below 90 mm. Hg would be unlikely to have toxemia.

CONCLUSIONS

1. A comparative study has been made of the TEAC blood pressure response during and after recovery from toxemia of pregnancy, in the prepartum and postpartum periods of normal term pregnancy, and in normal non-pregnant women.
2. In normal term pregnancy the TEAC blood pressure floor is strikingly low and rises to normal levels after delivery. In toxemia the TEAC floor is higher than normal and consistently falls to normal levels after recovery.
3. Since TEAC, by blocking the autonomic nervous system at the ganglia, eliminates neurogenic tone but does not lessen humoral tone, the results suggest that the hypertension of toxemia of pregnancy is supported by an excessive degree of humoral tone.
4. Because of the striking depressor response to TEAC in normal term pregnancy, it appears likely that neurogenic mechanisms are more active in normal pregnancy than in toxemia.
5. Clinical assay with TEAC may be a helpful aid in diagnosis of toxemia of pregnancy and in the evaluation of changes in severity during its course.

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URINARY EXCRETION OF AMINO ACIDS FOLLOWING THE RAPID INJECTION OF A SOLUTION OF AMINO ACIDS IN MAN^{1,2}

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The parenteral administration of partial and complete protein hydrolysates is accompanied by an appreciable excretion of the infused amino acids and polypeptides in the urine (1-5). The renal loss of amino nitrogen following parenteral administration may be as great as 28 times the loss from the administration of an equivalent amount of nitrogen as whole protein orally (6). If, in addition to such urinary losses, one or more of the essential amino acids were present in "limiting" amounts or were preferentially excreted, the amount of a protein just capable of maintaining nitrogen equilibrium when given orally might be insufficient when given intravenously as a hydrolysate (6).

The experiments reported here were undertaken to determine the extent of the renal loss of amino acids after their intravenous infusion. Determinations were made of the renal excretion of the eight amino acids essential for man (7) and of arginine and histidine, the urinary excretion of alpha amino nitrogen, and the blood alpha amino nitrogen following the intravenous administration to normal subjects of a 10 per cent solution of amino acids. The results indicate that the administration of protein or amino acids orally or intravenously, slowly or rapidly, and in large or small quantity is followed by the excretion in the urine of but a small portion of that administered.

MATERIALS AND METHODS

The amino acid mixture³ was made by the complete acid hydrolysis of casein, contains no peptides, is essen-

tially devoid of the dicarboxylic amino acids (glutamic and aspartic) and is supplemented with dl-methionine, dl-tryptophane, and glycine so that it contains the eight amino acids essential for man (7). In Table I the composition of the lot of the solution of amino acids employed in this study is shown and is compared to that of casein. The results are all based on analyses by microbiological assay (8-10).

The normal subjects selected for this study were medical students or interns. The urine was collected for one day prior to the infusions during which time the subjects ate *ad libitum*. The last two-hour urine collection (6 to 8 a.m.) of the 24-hour control period was saved separately, and thus represented a two-hour fasting specimen in contrast to the preceding 22-hour non-fasting collection. Each 500-cc. infusion of the 10 per cent solution of amino acids supplied 47 gm. of amino acids, 7.5 gm. of total nitrogen, and 6.0 gm. of alpha amino nitrogen, and was given without glucose to the subjects while fasting. Blood samples for plasma alpha amino nitrogen determinations were obtained prior to the infusion and at five-minute, one-hour, and four-hour intervals after the infu-

TABLE I
Comparison of the composition of the 10 % solution of amino acids with casein

	Amino acid solution	Casein†	Amino acid solution
	gm. per liter of 10% solution*	gm. per 100 gm.	500 cc. of 10% gm. per injection‡
Arginine	4.9	3.7	2.5
Histidine	3.1	2.8	1.6
Isoleucine	7.6	6.9	3.8
Leucine	15.6	10.1	7.8
Lysine	8.4	7.6	4.2
Methionine	6.4	2.8	3.2
Phenylalanine	5.3	5.3	2.7
Threonine	2.1	4.3	1.1
Tryptophane	0.9	1.2	0.45
Valine	6.2	6.2	3.1

* Determinations by microbiological assay (l-form) (8). Also contains: 0.9 gm. d-tryptophane, 2.5 gm. d-methionine, 0.2-0.5 gm. glutamic acid, 0.2 gm. tyrosine, 22.6 gm. glycine, less than 0.05 gm. aspartic acid, and (by difference) 7.0 gm. non-assayed amino acids per liter of 10 per cent solution. Total 10 "essential" amino acids (l-form) comprise 64 per cent of total amino acids in mixture.

† Averaged values of Stokes *et al.* (8), Hodson and Krueger (9), and Pearce *et al.* (10). Microbiological assay.

‡ Each 500 cc. infusion supplies 47 gm. of amino acids, 7.5 gm. of total nitrogen, and 6.0 gm. of alpha amino nitrogen.

¹ The expenses of this investigation were defrayed in part by a grant from Merck and Company, Inc., Rahway, New Jersey, to Harvard University.

² A preliminary report of this investigation was included in the Proceedings of the Thirty-Ninth Annual Meeting of the American Society for Clinical Investigation, May 5, 1947.

³ "Solution of Amino Acids Mixture, 10%," developed and distributed by Merck & Co., Inc., Rahway, N. J., who supplied the material used in this investigation.

sion had been completed. The urine was collected during this four-hour period,⁴ and separately over the next 20 hours. Food was withheld until four hours after the infusion, and was then ingested as for the preceding control day.

The alpha amino nitrogen was determined by the gasometric ninhydrin method as described by Hamilton and Van Slyke for plasma (11), and by Van Slyke, MacFadyen, and Hamilton for urine (12). All individual amino acid analyses in the urine were by microbiological assay (8). Since the unnatural isomers of methionine and tryptophane are not detected by microbiological assay (8), all values presented here are for the naturally occurring l-forms, although both d- and l-methionine are nutritionally available for man (13). We have, therefore, no data concerning the loss by renal excretion of the d-methionine and d-tryptophane infused in these subjects.

RESULTS

In Table II are shown the blood amino nitrogen values and the urinary excretion of alpha amino nitrogen following the infusion of 500 cc. of the 10 per cent solution of amino acids in the eight normal subjects. The solution of amino acids was infused at varying rates that fall roughly into three

⁴ This specimen was collected from the time the infusion started until four hours after its conclusion, and thus covered somewhat more than four hours' time. For purposes of calculation, it was considered a four-hour collection since the infusion time was short enough to make the error so incurred negligible.

rate periods of (a) 2 mgm. of nitrogen per kilogram per minute, (b) 6 mgm. of nitrogen per kilogram per minute, and (c) 10 mgm. of nitrogen per kilogram per minute. These infusion rates roughly correspond to the infusion of 50 gm. of amino acids to a 70 kgm. subject in (a) one hour, (b) 20 minutes, and (c) 10 minutes. All infusions supplied over 0.5 gm. of amino acids per kilogram of body weight. There were no reactions to the infusions save for transient nausea without vomiting in two subjects who received the solution at the most rapid rate.

The control non-fasting 24-hour urine alpha amino nitrogen excreted by these eight normal subjects averaged 158 mgm. daily (Table II). The extremes (120 and 199 mgm. daily) probably reflect the varied protein intake of their diets, estimated to range from 80 to 130 gm. daily. Per hour, these non-fasting values for urinary amino nitrogen averaged 6.6 mgm. (range 5.0 to 8.3) which is higher than the average fasting value of 4.3 mgm. per hour (range 3.7 to 5.7) as determined by the two-hour pre-injection urine collection in these subjects. The difference of 2.3 mgm. per hour or 55 mgm. daily can be considered the renal loss from the protein ingested by these subjects. An estimated average protein intake of 105 gm. daily would represent about 16.8 gm. of

TABLE II

Blood alpha amino nitrogen values and urine alpha amino nitrogen excreted after injection of 500 cc. of 10% solution of amino acids at varying rates of infusion in eight normal adult subjects

Rate period	Subject	Infusion rate	Blood alpha amino nitrogen				Urine alpha amino nitrogen									
			Control	Time after infusion			Control*	Time after infusion								
				5 min.	1 hr.	4 hrs.		0 to 4 hours			4 to 24 hours		Total 24 hours			
I	N. D. J. T.	mgm. N/ kgm./min.	mgm./100 cc. plasma				mgm./ 24 hrs.	excess mgm./ 4 hrs.†	per cent excreted‡	excess mgm./ 20 hrs.†	per cent excreted‡	excess mgm./ 24 hrs.†	per cent excreted‡	average per cent excreted‡		
		1.9 2.0	3.3 3.5	16.3 12.5	6.8 6.1	4.1 3.8	154 180	477 441	7.9 7.3	15 4	0.3 0.1	492 445	8.2 7.4	7.8		
II	W. D.	5.8	3.4	23.2	8.9	3.8	139	489	8.2	18	0.3	507	8.5	9.3		
	T. C.	6.4	4.2	24.7	7.4	4.2	199	715	11.9	2	0.0	717	11.9			
	A. G.	6.6	3.8	24.3	7.3	4.2	160	439	7.3	4	0.1	444	7.4			
III	F. E.	9.0	4.1	27.4	7.3	4.4	158	756	12.6	4	0.1	760	12.7	10.7		
	A. B.	9.6	3.3	22.8	6.1	3.1	120	469	7.8	32	0.5	501	8.3			
	J. S.	11.6	3.2	28.6	7.4	3.6	156	656	10.9	18	0.3	674	11.2			
Average 8 infusions		6.6	3.6	22.5	7.2	3.9	158	555	9.3	12	0.2	568	9.5			

* Mg. alpha amino nitrogen excreted while receiving the *ad libitum* diet.

† Mg. alpha amino nitrogen in excess of that excreted while receiving the *ad libitum* diet.

‡ Per cent of administered alpha amino nitrogen (Table I) excreted in urine.

TABLE III

Ten "essential" amino acids excreted in urine (mgm. per 24 hours) by eight normal adult subjects receiving ad libitum diets

Subject	Arg.	Hist.	Isol.	Leuc.	Lys.	Meth.	Phen.	Threo.	Tryp.	Val.
N. D.	9.9	152	17.3	7.0	37.0	3.8	8.6	21.6	13.0	7.7
J. T.	9.7	218	12.5	7.5	99.9	4.8	19.1	34.6	14.7	9.0
W. D.	8.0	91	11.2	3.3	87.2	4.4	7.6	14.6	9.7	6.2
T. C.	13.8	198	7.5	11.4	26.5	6.8	19.4	37.5	16.5	11.4
A. G.	12.8	169	11.3	10.9	42.9	5.7	28.0	16.4	12.9	8.7
F. E.	3.0	123	13.8	3.6	22.8	2.0	6.6	7.7	11.6	2.7
A. B.	9.0	133	23.6	7.4	20.4	3.8	10.8	15.0	9.2	7.4
J. S.	9.6	222	13.1	6.5	37.6	4.1	11.8	20.1	13.8	6.4
Average 8 normals	9.5	163	13.8	7.2	46.8	4.4	14.0	20.9	12.7	7.4

total nitrogen ($P \times 16$ per cent) and 13.4 gm. of amino nitrogen ($N \times 80$ per cent), or an excretion loss of amino nitrogen of approximately 0.4 per cent of that ingested. The urinary excretion of the 10 individual amino acids in the eight normal subjects on *ad libitum* diets is tabulated in Table III.

The fasting blood alpha amino nitrogen values averaged 3.6 mgm. per 100 cc. of plasma and are in

agreement with values previously found in this laboratory (14). The values showed a greater elevation the more rapid the injection five minutes after the infusion was completed, but the one-hour and four-hour post-injection values were quite comparable for all rates of infusion. Although the four-hour values fell to within normal limits, most were slightly above the pre-injection level and averaged 3.9 mgm. per 100 cc. of plasma. More

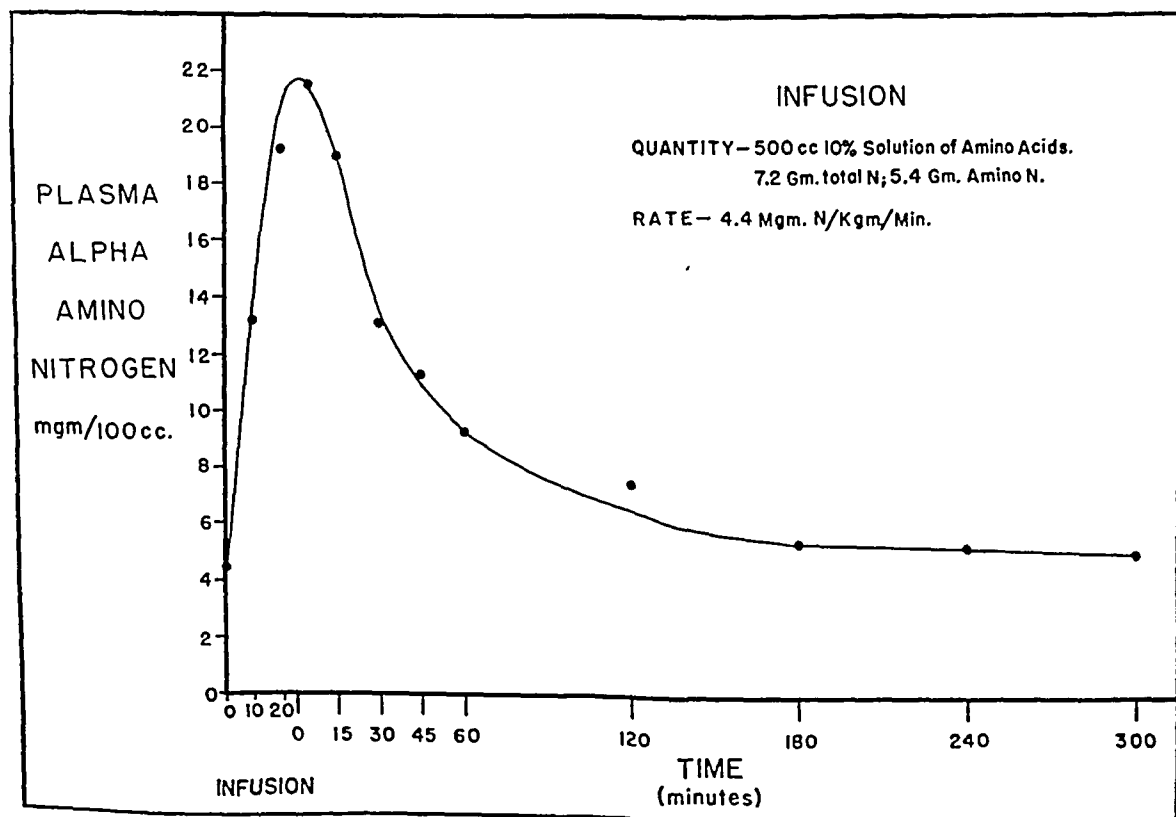


FIG. 1. CHANGES IN THE PLASMA ALPHA AMINO NITROGEN CONCENTRATION FOLLOWING THE INFUSION OF 500 CC. OF THE 10% SOLUTION OF AMINO ACIDS

TABLE IV

Ten "essential" amino acids excreted in urine in the 24 hours after injection of 500 cc. of 10% solution of amino acids at varying rates of infusion (eight normal adults)

	Slow rate (N. D., J. T.)		Moderate rate (W. D., T. C., A. G.)		Rapid rate (F. E., A. B., J. S.)		Average 8 infusions	
	mgm.*	per cent†	mgm.*	per cent†	mgm.*	per cent†	mgm.*	per cent†
Arginine	8.7	0.3	18.1	0.7	37.8	1.5	23.0	0.9
Histidine	206	12.9	190	11.9	231	14.4	209	13.1
Isoleucine	9.1	0.2	55.6	1.5	98.6	2.6	61.0	1.6
Leucine	45.7	0.6	172	2.2	281	3.6	182	2.3
Lysine	149	3.5	264	6.3	354	8.4	268	6.4
Methionine	34.4	1.1	88.7	2.8	169	5.3	105	3.3
Phenylalanine	43.1	1.6	112	4.2	175	6.5	119	4.4
Threonine	155	14.1	175	15.9	196	17.8	179	16.3
Tryptophane	22.7	5.7	25.7	6.4	33.8	8.4	28.0	7.0
Valine	42.1	1.4	105	3.4	196	6.3	124	4.0
Total 10 "essential" amino acids	716	2.3	1206	4.0	1772	5.8	1298	4.3

* Mgm. of amino acid in excess of that excreted while receiving the *ad libitum* diet (Table III).

† Per cent of administered amino acid (Table I) excreted in urine.

frequent determinations of plasma alpha amino nitrogen before, during, and following the infusion of the same quantity of amino acids in another subject are shown in Figure 1. It is apparent that the rapid decline in the blood amino nitrogen occurs in

the first hour after the infusion, while the subsequent decline toward normal is much slower.

The excess excretion of amino nitrogen above the control occurred within the first four hours following the infusion, averaged 9.3 per cent of

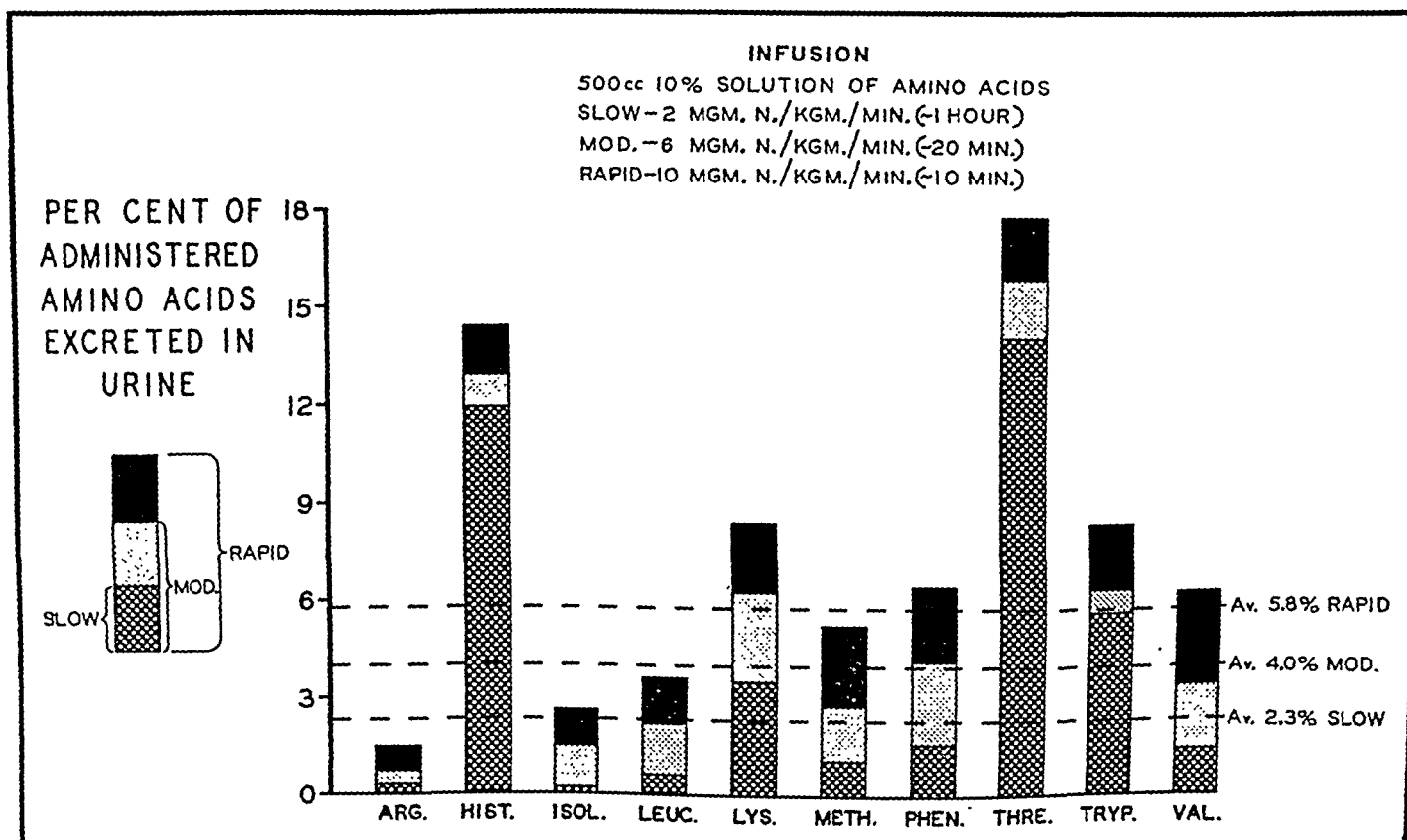


FIG. 2. COMPARISON OF THE PERCENTAGE OF ADMINISTERED AMINO ACIDS EXCRETED IN THE URINE FOLLOWING THE INFUSION OF 500 CC. OF THE 10% SOLUTION OF AMINO ACIDS AT THREE RATES

that infused, and coincided with the return of the blood amino nitrogen to within normal limits.⁵ The loss of amino nitrogen in the subsequent 20-hour collection period was negligible and averaged 0.2 per cent of that injected. The loss of amino nitrogen in the urine increased but little with more rapid infusions (Table II).

The loss in the urine of each of the infused 10 "essential" amino acids 24 hours following the injection at the different rates of infusion is tabulated in Table IV and plotted in Figures 2 and 3. Since the excess excretion above the control of each of the 10 infused amino acids occurred within four hours following the infusion with no further loss over the next 20 hours, only the total 24-hour

⁵ Two additional subjects in whom the urine was collected more frequently in the post-infusion period excreted approximately 90 per cent of the amino nitrogen lost within the first hour and the remaining 10 per cent during the next three hours.

post-injection values are listed. From 0.2 per cent to 14.1 per cent of the individual amino acids infused was excreted in the urine at the slow rate of infusion, and averaged 2.3 per cent for all 10 amino acids assayed. Thus the subjects excreted fairly large percentages of administered threonine and histidine, intermediate percentages of tryptophane and lysine, and smaller percentages of the other amino acids.

As the infusion rate increased from slow to moderate to rapid, an additional 1.7 per cent and 1.8 per cent, respectively, of the administered 10 "essential" amino acids was excreted. However, in contrast to the marked variation in the excretion loss of individual amino acids at the slow rate of infusion (0.2 to 14.1 per cent), their additional loss with more rapid infusions varied but little (from 0.0 to 2.0 per cent), as illustrated in Figure 2. Thus, for example, at the slow rate of infusion

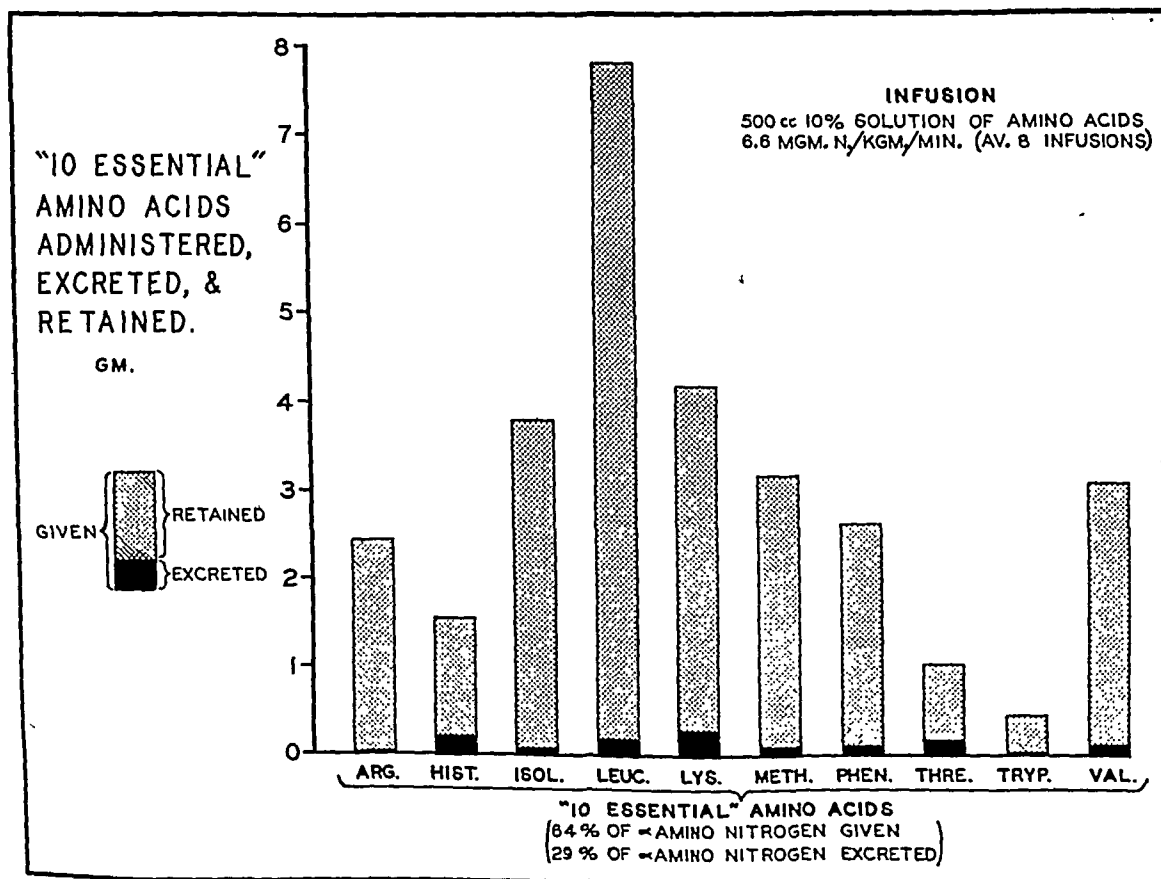


FIG. 3. RETENTION IN THE BODY AND URINARY EXCRETION OF THE 10 "ESSENTIAL" AMINO ACIDS FOLLOWING THE INFUSION OF 500 CC. OF THE 10% SOLUTION OF AMINO ACIDS

14.1 per cent of the administered threonine was excreted in the urine in contrast to but 0.6 per cent of the leucine given. Yet the additional loss of each of these amino acids with more rapid rates of infusion was approximately the same (1.8 and 1.9 per cent for threonine, and 1.6 and 1.4 per cent for leucine). Because of the marked variation in the percentage excretion of individual amino acids at the slow rate of infusion, it is apparent that a 2 per cent additional loss represents a considerable variation in the quantity of each amino acid excreted with more rapid rates. Thus, the additional loss of 1.8 per cent threonine and 1.6 per cent leucine at the moderate as compared to the slow rate of infusion represents for threonine only a small increase in the quantity excreted (from 155 to 175 mgm. or a 13 per cent increase), but a considerable increase in the quantity of leucine excreted (from 46 to 172 mgm. or a 275 per cent increase).

The patterns of the 10 "essential" amino acids administered intravenously, excreted in the urine, and (by difference) retained in the body are shown in Table V and Figure 4. The proportion of each of the "essential" amino acids in the mixture dif-

TABLE V

Pattern of the 10 "essential" amino acids administered, excreted and retained

	Administered*		Excreted†		Retained‡	
	mgm.	per cent of total	mgm.	per cent of total	mgm.	per cent of total
Arginine	2,450	8.1	23	1.8	2,427	8.4
Histidine	1,550	5.1	209	16.1	1,341	4.6
Isoleucine	3,800	12.6	61	4.7	3,739	12.9
Leucine	7,800	25.7	182	14.0	7,618	26.4
Lysine	4,200	13.9	268	20.6	3,932	13.6
Methionine	3,200	10.6	105	8.1	3,095	10.7
Phenylalanine	2,650	8.8	119	9.2	2,531	8.7
Threonine	1,050	3.5	179	13.8	871	3.0
Tryptophane	450	1.5	28	2.2	422	1.4
Valine	3,100	10.2	124	9.5	2,976	10.3
Total	30,250	100.0	1,298	100.0	28,952	100.0

* Table I.

† Table IV, averaged values of eight infusions.

‡ Quantity administered minus quantity excreted.

ferred considerably from that excreted in the urine. Those amino acids excreted in greater proportions than were present in the mixture (threonine, histidine, and to a lesser extent lysine and tryptophane) were the same amino acids lost in the greatest per-

PATTERN OF THE 10 "ESSENTIAL" AMINO ACIDS
ADMINISTERED, RETAINED, AND EXCRETED.

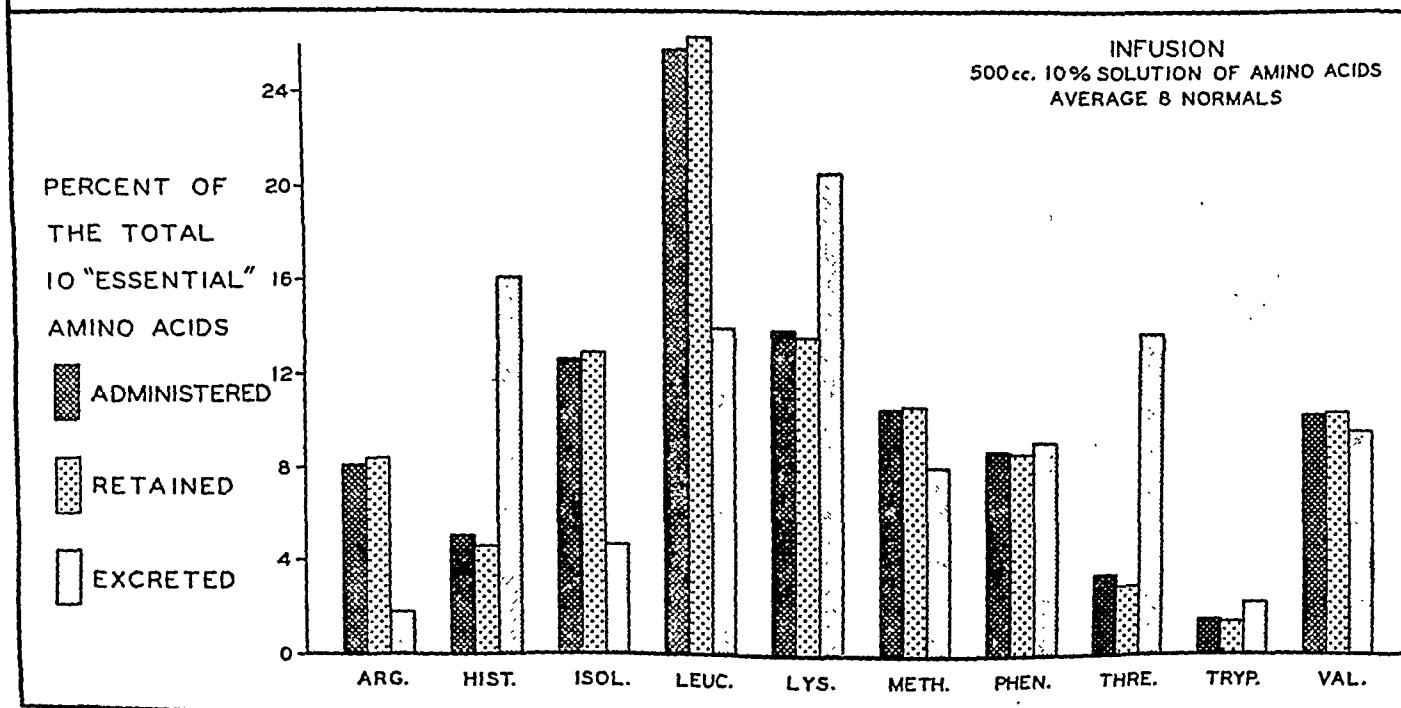


FIG. 4. COMPARISON OF THE PATTERN OF THE 10 "ESSENTIAL" AMINO ACIDS ADMINISTERED, RETAINED, AND EXCRETED FOLLOWING THE INFUSION OF 500 CC. OF THE 10% SOLUTION OF AMINO ACIDS TO EIGHT NORMAL SUBJECTS

centages of the amount given (Figure 2). Thus, for example, although threonine comprised only 3.5 per cent of the total 10 "essential" amino acids in the mixture infused, it contributed to 13.8 per cent of the total of these 10 amino acids excreted in the urine following the infusion, or a loss of 16.3 per cent of the threonine administered. Since only a small portion of the infused amino acids was lost in the urine (Figure 3), the pattern of the amino acids retained in the body resembled quite closely the pattern infused.

DISCUSSION

There is no ideal substitute for a well balanced oral diet. Nevertheless, there are instances in which supplemental or complete feeding by other routes is indicated. Whenever the parenteral route for protein nutrition is selected, the most nutritious and efficient preparation that is least objectionable to both patient and physician should be employed.

It was pointed out in a previous communication that the complete hydrolysate with which the studies reported here were conducted offers certain distinct advantages over other available preparations (15). Most evident is the reduced incidence of anorexia and nausea following administration, permitting infusion rates to be greatly increased. In addition, administration of this preparation is free from the uncertainties of the antigenicity of peptides and of the degree and rate of their utilization (4, 5). Fortunately even with hydrolysates containing peptides allergic reactions are but rarely encountered. Moreover, since this hydrolysate is a mixture of free amino acids for which the composition is known, it is possible to measure the urinary loss of the individual amino acids, as well as the total alpha amino nitrogen.

The results of the present investigation have shown that following infusions of 50 gm. of amino acids there is an average loss into the urine of from 1 to 16 per cent of the individual amino acids infused, of 4.3 per cent of the 10 "essential" amino acids injected, and of 9.5 per cent of the alpha amino nitrogen administered.⁶ The excretion of

both alpha amino nitrogen and individual amino acids was completed within four hours after the infusion by which time the elevated blood amino nitrogen had returned to within normal limits. The urinary loss of amino acids following infusion may be contrasted with that following the ingestion of an estimated 105 gm. of protein by our subjects in the day prior to infusion which only increased the excretion of amino nitrogen above the fasting level by 55 mgm. daily, or approximately 0.4 per cent of the ingested amino nitrogen. This value is identical with that observed to follow the oral ingestion of 50 gm. of whole casein (6).

The renal excretion of amino nitrogen was only slightly less at slow than at rapid rates of infusion. However, three of the subjects (T. C., F. E., and J. S., Table II) did excrete larger quantities of alpha amino nitrogen than did the others. Two of these (F. E. and J. S.) were of small stature and received proportionately greater quantities of amino acids per kilogram of body weight. This suggested that the urinary loss of amino nitrogen following infusions of a protein hydrolysate might correlate better with the quantity of material infused than with the rate of infusion. To substantiate this, the amino nitrogen excretion following 32 infusions of 500 cc. of the 10 per cent solution of amino acids was compared with that following 16 infusions of 1000 cc. administered to normal subjects on nitrogen balance studies. There was an average loss of 9.3 per cent (range 3.3 to 13.1 per cent) of the infused amino nitrogen in the first group and 14.0 per cent (range 8.5 to 18.7 per cent) in the second. Although in these two groups there was again no significant relationship between the rate of infusion and the urinary excretion of amino nitrogen, there was an increased excretion upon doubling the quantity given.

The significance and interpretation of the urinary excretion of amino acids has been the object of extensive investigation. All of the amino acids that have been studied are almost totally reabsorbed by the renal tubules at normal plasma concentrations (17, 18) so that normally only small quantities of amino acids are found in human urine. The values for the 10 "essential" amino acids excreted daily by our eight normal subjects while on *ad libitum* diets (Table III) are in general agreement with the published values obtained by others employing microbiological methods (19-21), al-

⁶ Because neither food nor glucose was supplied with the amino acid infusions, our values probably represent maximum urinary losses since optimum sparing of nitrogen (and presumably amino nitrogen) occurs only if adequate carbohydrate and protein are simultaneously provided (16).

though slightly higher perhaps because of the liberal protein diets of our subjects.

Following the infusion of the solution of amino acids all ten were found in greatly increased quantities in the urine during the four hours following the infusion, while none exhibited a "delayed" excretion over the next 20 hours. The excretion in the urine of the total of the 10 amino acids averaged 4.3 per cent of the quantity injected (Table IV), while that of the alpha amino nitrogen averaged 9.5 per cent of that given (Table II). The higher value for amino nitrogen probably reflects the urinary excretion of those amino acids contained in the hydrolysate but not assayed, for 36 per cent of the amino nitrogen in the solution administered consists of glycine and other non-essential amino acids, as well as d-tryptophane and d-methionine (Table I). Moreover, although the 10 "essential" amino acids comprised 64 per cent of the amino acids infused, they contributed to less than one-third of the total amino nitrogen excreted in the urine (Table VI). Thus the subjects appeared to preferentially retain the essential amino acids; and, conversely, the dispensable amino acids were more freely excreted in the urine. As a group, the 10 "essential" amino acids were retained to the greatest degree at the slowest rate of infusion, and were relatively less well conserved with more rapid infusions.

There was a marked variation observed in the percentage excretion in the urine of the individual amino acids infused (Table IV and Figure 2). It is not possible from data available to ascertain why

TABLE VI

Relation between rate of infusion and quantity of amino acids excreted in urine in the 24 hours after injection of 500 cc. of 10% solution of amino acids

Infusion rate	Total amino acids excreted*	Total 10 "essential" amino acids excreted†	Amino acids not assayed (by difference)	10 "essential" amino acids
	gm.	gm.	gm.	per cent of total
Slow	3.66	0.72	2.94	20
Moderate	4.34	1.21	3.13	28
Rapid	5.04	1.77	3.27	35
Average all rates	4.44	1.30	3.14	29

* Calculated from α -amino nitrogen excretion values, Table II, assuming α -amino nitrogen to be 80% of total nitrogen, and total nitrogen to be 16% of amino acids.

† Determined by microbiological assay, Table IV.

each of the amino acids was not excreted in the urine in the same proportion as administered (Table V and Figure 4). At least three explanations exist: (1) differences in the quantity of amino acids infused as compared to their requirements by the body; (2) differences in the renal clearance of amino acids; and (3) differences in the rate of removal of amino acids from the blood stream by tissues. With each increase in the rate of infusion, however, an almost equivalent additional per cent of each amino acid administered was excreted in the urine. Thus with more rapid infusions a constant proportion of that given was lost. The question may now be asked: Does the increased excretion in the urine of amino acids when parenteral rather than oral administration is employed significantly decrease the quantity of amino acids available to the body for metabolic purposes? This might be true if protein containing minimum quantities of each of the essential amino acids administered orally in an amount just sufficient to maintain nitrogen equilibrium were injected as a hydrolysate intravenously. Thus it was observed in a previous study (6) that a protein hydrolysate which contained minimum amounts of phenylalanine maintained nitrogen equilibrium when administered orally, but was incapable of sustaining nitrogen balance when given parenterally until additional phenylalanine was provided. Moreover, were an essential amino acid preferentially excreted into the urine following intravenous administration, the quantity available for metabolic purposes would be further reduced. In this regard it was observed that greater proportions of the administered threonine, histidine, and to a lesser extent lysine and tryptophane were excreted than of the other six amino acids. It is not possible to decide from the available data whether this represents preferential excretion rather than simply loss of these amino acids above the body's requirements. If amino acids are being preferentially excreted, additional quantities might have to be provided.

Further, it might be asked: To what extent is the nutritive value of the hydrolysate decreased by rapid or by large infusions? As illustrated in Figures 3 and 5, the administration of protein or amino acids orally or intravenously, slowly or rapidly, and in large or small quantity, is followed by excretion in the urine of but a small portion of

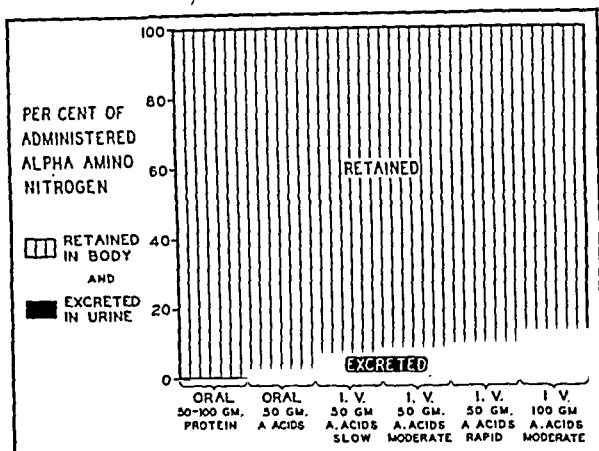


FIG. 5. COMPARISON OF THE PERCENTAGE OF ALPHA AMINO NITROGEN EXCRETED FOLLOWING THE ORAL ADMINISTRATION OF PROTEIN OR AMINO ACIDS AND THE INTRAVENOUS INFUSION OF AMINO ACIDS AT VARYING RATES AND IN VARYING AMOUNTS

the amino acids given. Although the loss following the parenteral administration of the protein hydrolysate was approximately 25 times greater than when protein was ingested orally, the largest quantity of amino acids excreted was so small in terms of grams of protein that the body retained for metabolic purposes more than 85 per cent of the protein given. Infusions of smaller quantities of the solution of amino acids than employed in this study might be expected to result in a smaller urinary loss of amino acids, but this small saving would not be of value since the quantity thus infused would be less than the need of the individual. Similarly, very slow infusions might result in a saving to the body of an additional few grams of the infused amino acids, but the inconvenience so incurred by both patient and physician would hardly appear to justify this practice since one of the advantages of this hydrolysis is the rapidity of infusion attainable in both animals (22) and man (15) with but few untoward effects.

SUMMARY

The 10 per cent solution of amino acids employed in this study was prepared by the complete acid hydrolysis of casein, contains no peptides, is devoid of the dicarboxylic amino acids (glutamic and aspartic) and is supplemented with dl-methionine, dl-tryptophane, and glycine. It contains the "essential" amino acids required by man.

Casein hydrolysate in the amount of 500 cc. (50 gm. amino acids) was infused without glucose to eight normal subjects, while fasting, at three rates of administration: approximately 2 mgm., 6 mgm., and 10 mgm. of nitrogen per kilogram per minute, representing infusion times of approximately one hour, 20 minutes, and 10 minutes, respectively. The plasma alpha amino nitrogen was determined prior to the infusion, and at five-minute, one-hour, and four-hour intervals after the infusion was completed. Determinations of the alpha amino nitrogen and of the 10 "essential" amino acids (microbiological assay) were made on urine obtained prior to the infusion, in the first four hours and in the next 20 hours after the infusion was completed.

The infusions were well tolerated. Two subjects who received the 50 gm. of amino acids intravenously in approximately 10 minutes noted transient nausea near the end of the infusion.

The ingestion of an estimated 105 gm. of protein in the diets of the eight normal subjects during the day prior to infusion only increased the excretion of amino nitrogen above the fasting level by 55 mgm. daily, or approximately 0.4 per cent of the ingested amino nitrogen. The average excretion of the 10 "essential" amino acids on these *ad libitum* diets was: arginine 9.5; histidine 163; isoleucine 13.8; leucine 7.2; lysine 46.8; methionine 4.4; phenylalanine 14.0; threonine 20.9; tryptophane 12.7; and valine 7.4 mgm. per 24 hours.

The blood alpha amino nitrogen values for the eight subjects averaged 3.6 mgm. per 100 cc. of plasma initially, rose to 22.5 mgm. per cent five minutes after the infusion, rapidly fell to 7.2 mgm. per cent one hour later, and returned to within normal limits by four hours although most values were slightly above the pre-injection level and averaged 3.9 mgm. per cent.

There was an average loss from excretion into the urine of 1 to 16 per cent of the individual amino acids infused, 4.3 per cent of the total 10 "essential" amino acids injected, and 9.5 per cent of the amino nitrogen administered. The increased excretion of both amino nitrogen and individual amino acids was completed within four hours after the infusion by which time the elevated blood amino nitrogen had returned to within normal limits.

The excretion of amino nitrogen in the urine increased little with more rapid rates of infusion but correlated more closely with the size of the infusion. The "essential" amino acids in the infusion mixture were preferentially retained by the subjects while the dispensable amino acids were more freely excreted in the urine. The greatest retention of the "essential" amino acids was observed at the slowest rate of infusion.

The individual amino acids were not excreted in the urine in the same proportion as administered, so that fairly large percentages of administered threonine and histidine were excreted, intermediate percentages of lysine and tryptophane, and smaller percentages of the other amino acids. With each increase in the rate of the infusion, however, the additional excretion of all the "essential" amino acids more closely resembled the composition of the amino acids infused.

The administration of protein or amino acids orally or intravenously, slowly or rapidly, and in large or small quantity is followed by excretion in the urine of but a small portion of the amino acids given so that the body retains for metabolic purposes most of that administered.

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A METHOD FOR THE QUANTITATIVE DETERMINATION OF THE CEPHALIN-CHOLESTEROL FLOCCULATION REACTION¹

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The cephalin-cholesterol flocculation reaction of Hanger (1) has been found to be an extremely useful test in routine clinical work because of its simplicity and the very small number of false positive reactions in normal cases. Although positive reactions have been obtained in such diseases as malaria, pneumonia, septicemia, infectious mononucleosis, etc. (1-3), the test has found its widest application in the study of liver disease. A positive reaction in most cases indicates parenchymal cell injury and the test has been found most useful in the early diagnosis of liver disease (4, 5). Since the degree of flocculation of the cephalin-cholesterol emulsion by the patient's serum parallels the severity of the disease, serial determinations of this test have been found useful as an aid in the prognosis of various diseases (1, 4, 6). At the present time, results are expressed in a qualitative manner, *i.e.*, 0 to 4+, and since negative values are often obtained before the jaundice has completely subsided, the test is of little diagnostic value late in the course of the disease (7).

Investigation of the flocculated material from positive cases has shown it to be a complex composed of a protein material, *i.e.*, gamma globulin, attached to the cephalin-cholesterol emulsion (8). The probability of alpha and beta globulin from hepatitis sera also taking part in this reaction has recently been mentioned in the literature (9). The probable mechanism of the cephalin-cholesterol reaction including the inhibiting role of the albumin fraction has been described by Moore *et al.* (10) and is based on experimental studies made with electrophoretically separated fractions of normal and diseased human sera.

All the previous investigators have been concerned mainly with the nature of the protein part of the complex. The present investigation deals

with the quantitative determination of the cholesterol content of the centrifuged material after the completion of the usual qualitative test.

PROCEDURE

The cephalin-cholesterol emulsion was prepared from the commercial product supplied by the Difco Laboratories, Inc., Detroit, Michigan, Lot No. 388716. The emulsion was prepared and the test run exactly as described by Hanger (1) except that the centrifuge tubes were kept in a dark closet as suggested by Neefe and Reinhold (11). Readings were made after 24 hours' standing and listed as 0, \pm , +, ++, +++, and +++++, using Hanger's criteria of reading the results. No readings were made after 48 hours' standing as it was found that the sensitivity of the emulsion was such that about 10 per cent of the normal sera run gave + or ++ readings at that time (12). A saline blank, a known normal serum and a known +++++ serum were set up with each run.

After the completion of the qualitative test, the centrifuge tubes containing the samples were centrifuged at 3000 r.p.m. for exactly 15 minutes and the supernatant fluid was carefully poured off so as not to disturb the precipitate. The lips of the inverted tubes were allowed to drain on a piece of filter paper for a few seconds, the top inside surface of the tube wiped carefully with a clean piece of filter paper, and the tubes replaced upright in the rack. Approximately 0.1 ml. of distilled water was added to each centrifuge tube, followed by 4.5 ml. of acetic anhydride-dioxane mixture. The tubes were then placed in a boiling water bath and heated for 30 minutes or longer with occasional shaking. The tubes were then removed and cooled to room temperature and the contents transferred quantitatively to a colorimeter tube (or to a 5-ml. glass-stoppered graduated cylinder where individual cuvettes are used) graduated at 5.00 ml., with the aid of several small portions of the acetic anhydride-dioxane mixture and the contents diluted exactly to mark. The remainder of the procedure for the determination of the total cholesterol content of the precipitate is that described by Saifer and Kammerer (13). Small amounts of protein which may be washed into the colorimeter tube have a negligible effect on the accuracy of the method.

The results are expressed in *cholesterol units* (which are equivalent to the mg. per cent of total cholesterol for a 0.20-ml. serum sample) after subtraction of the saline blank value.

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TABLE I

Relationship of qualitative cephalin-cholesterol flocculation results to quantitative cholesterol unit values

Qualitative values	No. of cases	Range of quantitative cholesterol units	Average cholesterol unit value
Negative (All cases)	48	0-204	47
+	9	122-258	188
++	12	129-332	253
+++	13	243-473	407
++++	13	410-633	529
Negative (Normals only)	20	0-103	43

RESULTS

Some 20 normal sera and 80 sera from hospital patients, including a large group of cases with liver disease, were run by the procedure described above. The qualitative results obtained by the Hanger method, the number of cases in each group, the range of quantitative cholesterol units for each group and the average quantitative cholesterol value for each qualitative group are given in Table I. The results obtained indicate that there is a direct quantitative relationship between the quali-

TABLE II

Reproducibility of quantitative cholesterol unit values obtained for normal and pathological sera

Patient and diagnosis	Qualitative values	Quantitative cholesterol units	Quantitative cholesterol units (average)	Probable error of a single determin. cholest. units
No. 1 (Normal)	0 0 0	17 30 23	23	4.4
No. 2 (Normal)	0 0 0	11 6 8	8	4.2
No. 3 (Unknown)	+	205	206	3.0
	+	210		
	+	202		
No. 4 (Infectious hepatitis)	3+ 3+ 3+	485 476 478	480	3.7
No. 5 Cirrhosis	4+ 4+ 4+	642 648 636	642	4.1

tative results by the Hanger method and the quantitative cholesterol unit values of the centrifuged complex.

To check the reproducibility of the method, a number of normal and pathological sera were run in triplicate by the above method. The quantitative cholesterol units obtained together with the errors involved in the procedure are given in Table II. In Table IV, the reproducibility of the method is again shown by the relatively constant cholesterol unit values obtained on repeated analysis, at intervals, of the author's own serum. The data in Table III are presented to show the effect

TABLE III

Effect of using different cephalin-cholesterol emulsions on the quantitative cholesterol unit values obtained with the same sera.

Patient and diagnosis	Emulsion	Qualitative values	Quantitative cholesterol units	Cholesterol unit value (Average)	Probable error of a single determin. cholest. units
No. 8 Normal	I	0	0	4	2.8
	II	0	7		
	III	0	5		
No. 11 Normal	I	0	0	2	1.4
	II	0	4		
	III	0	2		
No. 12 Unknown	I	2+	302	305	9.3
	II	2+	318		
	III	2+	294		
No. 5 Cirrhosis	I	4+	531	530	19.6
	II	4+	558		
	III	4+	502		

on the same sera, normal or pathological, of different cephalin-cholesterol emulsions. Two of these emulsions, I and II, were prepared from the same lot of Difco antigen while emulsion III was prepared from another lot from the same company. The results of these experiments together with the errors involved in using the different emulsions are given in Table III.

To determine whether this procedure could be used in the prognosis of liver disease, a number of typical cases were followed by means of serial quantitative determinations at frequent intervals. The data obtained in these runs are presented in Table IV, together with other pertinent data, *e.g.*, thymol turbidity, bromsulphalein, icteric index, etc., relating to the clinical condition of the patient.

TABLE IV

A comparison of serial qualitative and quantitative cephalin-cholesterol flocculation tests as an aid in the prognosis of liver disease

Patient and diagnosis	Date of test: 1/8	1/13	1/16	1/18	1/22	1/27	1/29	2/3	2/10	2/17	2/25
No. 4 Infectious hepatitis	4+ 516 II = 16	4+ 493	4+ 443	3+ 430	3+ 371	3+ 367 TT = 11	—	—	3+ 377 TT = 9	2+ 359 TT = 9	1+ 149 TT = 6
No. 5 Cirrhosis	4+ 483 Brom = 32	4+ 540	4+ 510	4+ 484					4+ 642 TT = 6		
No. 6 Infectious hepatitis	—	3+ 399	3+ 415 Brom = 4.5 II = 8.6	—	3+* 461 Brom = 0.5 II = 6.3	—	3+ 523 TT = 3	3+ 538 TT = 3	3+ 492 TT = 3	3+ 482	2+ 342
No. 7 Infectious hepatitis	3+ 355	—	—	0 118 TT = 9	—	—	—	1+ 155 Bil = 3.0	2+ 273 TT = 7		
No. 8 Normal	—	—	0 4	0 2	—	0 4	—	—	—	0 6	—

0 = negative cephalin-cholesterol readings.

TT = Thymol Turbidity units.

II = Icteric Index.

Bil = total Bilirubin (mg. per cent).

Brom = Bromsulfalein (per cent retained).

* Patient hospitalized.

To determine whether cholesterol played a similar role in the thymol flocculation test of Neefe (14), a number of normal and abnormal sera were treated with the thymol reagent of MacLagan (15) and after measurement of the thymol turbidity, the contents were transferred to centrifuge tubes and allowed to stand for 24 hours in a dark closet. The thymol flocculation readings were then made and the contents centrifuged at 3000 r.p.m. for 15 minutes. The remainder of the procedure was performed exactly as described above for the cephalin-cholesterol flocculation reaction. The re-

sults obtained in this run are given in Table V. Only slight differences between the cholesterol content of negative and strongly positive thymol flocculation reactions are obtained upon analysis of the centrifuged material, as compared to the very large differences obtained for the cephalin-cholesterol precipitates of the same sera.

TABLE V

Relationship of thymol flocculation and cephalin-cholesterol flocculation readings to quantitative cholesterol units of centrifuged complex

Patient and diagnosis	Cephalin-cholesterol values—qualitative	Cephalin-cholesterol values—quantitative	Thymol flocculation values—qualitative	Thymol flocculation values—quantitative
No. 5 Cirrhosis	4+	483	3+	32
No. 9 Acute hepatitis	4+	410	0	2
No. 10 Ulcerative colitis	3+	329	0	16
No. 8 Normal	0	7	0	0

TABLE VI

*Effect of dilution with physiological saline of normal and pathological sera on the cephalin-cholesterol flocculation test as determined by the qualitative and quantitative procedures**

Quantity of serum ml.	Patient and diagnosis					
	No. 8	No. 13	No. 5	No. 4	No. 12	No. 7
	Normal	Normal	Cirrhosis of liver	Hepatitis	Unknown	Hepatitis
0.20	2(0)	96(0)	606(4+)	430(3+)	289(2+)	258(1+)
0.10	183(1+)	415(3+)	657(4+)	609(4+)	418(3+)	335(3+)
0.05	604(4+)	658(4+)	695(4+)	734(4+)	684(4+)	731(4+)
0.02	701(4+)	653(4+)	473(4+)	733(4+)	695(4+)	738(4+)
0.01	707(4+)	301(4+)	325(4+)	725(4+)	616(4+)	757(4+)
0.005	430(4+)	61(4+)	212(4+)	450(4+)	279(4+)	165(4+)

* Note: Figures in parentheses give the qualitative cephalin-cholesterol flocculation readings.

All cholesterol unit values were calculated as if each tube contained 0.20 ml. of serum. The true cholesterol unit values can be obtained by multiplying the values in the table by the factor $\left(\frac{0.20}{\text{Quantity of serum used}}\right)$.

Bruger (16) and Mirsky and von Brecht (17) have shown that the dilution of both normal and pathological sera lead to an increase in the degree of flocculation by the Hanger method. It was decided to reinvestigate the dilution phenomena by the quantitative procedure so as to determine whether the proportion of serum to emulsion presently used in the qualitative method is the most suitable one for the purpose of distinguishing normal from abnormal sera. The results obtained in this run are given in Table VI.

ANALYSIS OF RESULTS

The 95 cases given in Table I furnish ample evidence in support of the fundamental observation of this paper, that there is a quantitative relationship between the degree of flocculation and the cholesterol content of the centrifuged complex. All normal individuals, who gave negative qualitative results, gave quantitative cholesterol unit values of 103 or less with an average value of 43 cholesterol units. Negative qualitative values which gave more than 100 cholesterol units were usually known liver disease cases in the convalescent stage of the disease when the Hanger test is known to become negative while the thymol turbidity values still remain elevated (18).

Between the various qualitative groupings, *i.e.*, 0 to 4+, there is a spread of between 65 to 154 cholesterol units between each group. The overlapping values for each of the various groupings given under "range of cholesterol units" is to be expected because of the personal error involved in deciding the exact qualitative reading of any given determination. The average cholesterol value for each group therefore furnishes a better index for the experimental fact that the quantitative cholesterol unit value increases with the degree of flocculation.

The data given in Table II illustrate the reproducibility of the quantitative method for the same normal or pathological sera when determined with the *same* cephalin-cholesterol emulsion. While the percentage error is greatest for the negative qualitative values and is least for the higher qualitative values, the probable error of any single determination remains remarkably constant over the entire range at about ± 4 cholesterol units.

The effect of using *different* cephalin-cholesterol

emulsions on the quantitative cholesterol unit values obtained with the *same* sera is shown by the data which are given in Table III. Emulsion II seems to give consistently the highest values for each of the sera while Emulsion III gives the lowest values. Unlike the results obtained in Table II, the probable error of a single determination appears to increase markedly with the qualitative reading and for a 4+ qualitative result, the error is approximately ± 20 cholesterol units although it is only about ± 4 units for a negative qualitative test.

That reproducible results can be obtained with the same sera when different emulsions are used are shown by the data on the author's own serum (No. 8.) in Table IV. The cephalin-cholesterol emulsions were freshly prepared on the date given together with several saline blanks. The quantitative cholesterol values were obtained after subtraction of the *lowest* saline blank. The values obtained for the same individual are remarkably consistent, giving an average cholesterol value of 4 units, and are all well within the expected error of ± 4 cholesterol units.

In Table IV, data are presented to illustrate the use of the method to follow the clinical course of a number of selected cases by means of serial determinations by the quantitative procedure. The same lot of Difco cephalin-cholesterol antigen was used for all of these studies although a fresh emulsion was prepared for each day's run.

Patient No. 4 represents a typical case of infectious hepatitis with an uneventful recovery on prolonged bed-rest with a high-protein, high-carbohydrate diet. The quantitative cholesterol values show a continuous decrease, except in one instance, and parallel quite closely the decrease in thymol turbidity values. It should be noted that the decrease in the cholesterol unit value anticipates in each instance the decrease in the qualitative value. In the one instance where there is a slight rise, *i.e.*, from 367 to 377, instead of a fall in the quantitative value, the change is 10 units whereas it has been previously mentioned that changes of less than 20 units were not to be considered as significant because of the difficulty in obtaining a reproducible emulsion.

Patient No. 5 represents a typical case of cirrhosis of long duration and has consistently shown a bromsulfalein retention value of about 30 per

cent. This patient has repeatedly given 4 + qualitative cephalin-cholesterol flocculation values with occasional 3 + values and relatively normal thymol turbidity values over a period of about a year. The quantitative cholesterol unit values obtained indicate the possibility of a rhythmic pattern for this disease which may parallel periods of increasing or decreasing parenchymal cell injury.

Patient No. 6 represents a recurrent case of infectious hepatitis which was found upon routine testing previous to hospitalization. Although the qualitative readings remained almost constantly at 3 + for the entire period of observation, the quantitative readings show a progressive rise followed by a decline after a period of hospitalization and treatment. In this case all other liver function tests were found to be normal.

Patient No. 7 illustrates a case of infectious hepatitis which went from a 3 + to a negative cephalin-cholesterol qualitative value in a relatively short space of time. It should be noted that the quantitative cholesterol value remained above 100 units and the thymol turbidity value was still considerably elevated, indicating that the disease was active. The patient was permitted to resume activity and suffered a relapse as indicated by the increasing qualitative and quantitative cephalin-cholesterol flocculation values.

The data shown in Table V indicate that the thymol flocculation test does not lend itself to the same quantitative differentiation by means of cholesterol determinations as does the cephalin-cholesterol flocculation test. The differences between a 3 + flocculation and a negative serum, as measured in cholesterol units, were so slight as to be within the experimental error of the method. This furnishes additional experimental evidence of the differences between the two tests in addition to that given by Recant *et al.* (19).

The data shown in Table VI on the effect of dilution with physiological saline of normal and abnormal sera on the cephalin-cholesterol flocculation test are a good example of the use of the quantitative method as a means of studying the various factors which influence the Hanger reaction. All the sera tested, both normal and pathological, exhibit an increase in the degree of flocculation with increasing dilution of the sera until a maximum value is reached, after which continued dilution causes a rapid decline in the amount

of the flocculated material as measured in quantitative cholesterol units. It should be noted that it would be difficult, if not impossible, to draw similar conclusions from the qualitative data as given in Table VI, as in each instance when the quantity of sera used is less than 0.05 ml., the supernatant fluid is water clear and would be read a 4 + by the Hanger method although the actual amount of precipitate may vary markedly. No evidence could be found in the literature to justify the proportions of serum, saline and emulsion presently used in the Hanger test. The data in Table VI show that the greatest differences between normal and pathological cases are obtained for 0.20 ml. of serum and that the proportions of serum, saline and emulsion chosen by Hanger are the best ones for the purpose.

The data in Table VI also appear to confirm the views of Moore, Pierson, Hanger and Moore (10) of the presence of a reactive gamma globulin and an inhibitory substance in the albumin fraction in all sera whether normal or abnormal. Dilution at first appears to dilute the inhibitory substance to a greater extent than the gamma globulin so that there is at first an increase in the amount of flocculated material until a point is reached where further dilution causes a decrease in the amount of the flocculated material. It should be noted that patient No. 5 with the highest qualitative result (4 +) is the first to show a decreased quantitative value on dilution, although the same effect is not exhibited by the other positive sera as compared to the normal sera.

DISCUSSION

The fundamental observation of this paper is the quantitative relationship between the degree of flocculation and the cholesterol content of the centrifuged complex. This is not as surprising as may appear at first because the cephalin-cholesterol emulsion may be considered to act as a vehicle to whose surface the *active* gamma globulin attaches itself in stoichiometric proportions to form the flocculating material. For any given emulsion, it is to be expected that the cholesterol content of the flocculated material would be as true a quantitative index of the *active* gamma globulin as would be its protein nitrogen or its phospholipid content. Hanger (1) has shown that there is an increase in

the protein N in the flocculated material from a positive serum as compared to a blank containing only the emulsion. No experimental work has been performed as yet on the cephalin content of the complex.

The main experimental difficulty which has been encountered in both the qualitative and quantitative method is the difficulty of reproducing the cephalin-cholesterol emulsion or maintaining the stability of the emulsion once it is prepared. The saline blank containing the emulsion, but no serum, has given results varying from 20 to 70 cholesterol units. Occasionally an entire run would exhibit supersensitivity where all the normal controls showed flocculation; at other times a run would show almost complete insensitivity with known 4+ sera exhibiting little or no flocculation. In each run described above, known normal and 4+ sera were always included as checks on the proper sensitivity of the emulsion. The pitfalls of the Hanger flocculation test have been discussed in some detail by Mateer (20). However, when the above precautions have been taken, the results obtained were excellent in that all normal individuals, giving a negative qualitative test, usually gave quantitative values below 100 units, while those showing even the slightest degree of flocculation, gave values much greater than 100 units as shown in Table I.

The above data also suggest that each normal individual has his own *active* gamma globulin level and that this value tends to remain fairly constant as long as the individual remains healthy. For example, the author's own cholesterol unit value, as shown in Table IV, has been about 4 ± 4 cholesterol units while that of one of the laboratory technicians has consistently been 95 ± 5 cholesterol units. The possibility of establishing an accurate range of cholesterol unit values for all normal individuals and a definite value for a single individual may be one of the main advantages of the quantitative procedure over the original qualitative method.

By the term "*active* gamma globulin," as used in the above discussion, is meant that portion of the gamma globulin which is *not* inhibited by a substance present in the electrophoretically separated albumin fraction, as explained by Hanger (21), and which is free to combine with the cephalin-cholesterol emulsion. A small amount of such

"*active* gamma globulin" is present in almost all normal sera as distinguished from the *absolute* amount of gamma globulin present in the normal sera. A number of investigators have shown that there is an increase in the absolute value of the gamma globulin and a decrease in the albumin fraction in cases of liver disease (8, 10) and malaria (22) when these fractions are separated electrophoretically. It is these changes which are determined by the cephalin-cholesterol flocculation reaction.

It should be emphasized that the quantitative cephalin-cholesterol test is not being advocated as a substitute for, or refinement of, the Hanger test. The Hanger test results parallel those of the quantitative test, and since the qualitative test is performed in its entirety before the quantitative test is started, the qualitative results will generally suffice for routine clinical diagnostic or prognostic results. Since a single unit change, *e.g.*, from 1+ to 2+, is equivalent to a change of 100 or more cholesterol units in the quantitative test, the test should find its greatest usefulness in studying and measuring accurately the many factors which influence the Hanger reaction so as to lead to an improved test. The data given in Tables II, IV and VI present a clear indication of the advantages that the quantitative test has over the qualitative test for this purpose. In addition to the effect of the dilution of sera on the Hanger reaction, such factors as methods of stabilizing the cephalin-cholesterol emulsion by means of dispersing agents, effect of heating sera at various temperatures, effect of time of standing of sera, effect of variation of temperature at which the flocculation reaction occurs, the use of human gamma globulin as a standard for the reaction, and the effect of various protein fractions on the reaction, etc., are presently under investigation and will be reported in subsequent papers.

It should be further emphasized that, while an attempt was made to select typical cases as representative of a particular disease, the extremely small number of clinical cases does not warrant the drawing of any general conclusions about any disease. They are presented here in the hope that some clinicians will be sufficiently interested in applying the method to a sufficient number of clinical cases so as to establish definite patterns for such diseases. The quantitative test should have its

greatest clinical value in following clinical cases into the convalescent stage and, because of the relatively slow response of hepatitis and other liver disease cases to treatment, to the investigation of new methods for the treatment of these diseases.

SUMMARY AND CONCLUSIONS

1. The present investigation deals with the quantitative determination of the cholesterol content of the centrifuged protein-cephalin-cholesterol complex after completion of the qualitative Hanger test.

2. The quantitative cholesterol unit values (after subtraction of the saline blank) of more than 100 normal and pathological sera were determined by the procedure described and the following conclusions were drawn from these results:

- a. A direct quantitative relationship exists between the qualitative (Hanger) results and the quantitative cholesterol units.
- b. Negative qualitative results gave a wide range of quantitative cholesterol units, *i.e.*, from 0 to 204 units, with those of known normals usually being less than 100 units (average value equals 43 units).

3. Positive qualitative values, *i.e.*, from 1 + to 4 +, gave cholesterol unit values greater than 100 units. The clinical course of several liver disease cases has been followed by means of serial determinations with the quantitative method. Changes can be detected by this method even when no changes are observed by the Hanger procedure.

4. When the same emulsion is used, the probable error of a single determination in a series has been found to be ± 4 cholesterol units. With different emulsions, the probable error of a single determination varies from ± 3 units for a negative qualitative test to an error of ± 20 units for a 4 + qualitative result.

5. Experimental evidence is presented to show that the thymol flocculation reaction does not lend itself to quantitative differentiation by means of cholesterol determinations as does the cephalin-cholesterol flocculation test.

6. The quantitative cephalin-cholesterol flocculation test should find its greatest value in providing the means of accurately determining the

various factors which influence the Hanger reaction and for determining the efficiency of new treatments for liver and other diseases.

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THE INTRAVENOUS GLUCOSE TOLERANCE TEST IN PREGNANCY¹

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Since the introduction of the glucose tolerance test as a diagnostic aid the glucose has classically been administered by mouth. By this procedure the pregnant women may exhibit a normal tolerance to glucose or may in many instances exhibit a decreased tolerance to glucose (1-4). Selman (3), for instance, reports 22 out of 47 patients studied by him to show high, prolonged or high and prolonged blood glucose levels when judged by certain criteria. Hurwitz and Jensen (4) find normal fasting blood sugar levels and normal peaks, but 81 per cent of their pregnant series showed high blood sugar values at two hours. From these data these authors conclude that pregnancy exerts a deleterious effect on carbohydrate metabolism.

The disadvantages of the oral route of administration of the glucose are many and they have been pointed out by several authors (5-9). These disadvantages have led to the use of the intravenous route of administration (8, 9) as a better test of the ability of the subject to metabolize glucose. In pregnancy all the objections to the oral route of administration apply as they do in the non-pregnant individual. In addition, alterations in the gastrointestinal tract peculiar to pregnancy might complicate the picture further.

In view of these considerations we have determined the tolerance of pregnant women to intravenously administered glucose. The over-all procedure employed is essentially the same as the one described by Lozner, Winkler, Taylor and Peters (9) who have determined the tolerance of non-pregnant individuals to 25 grams of intravenously administered glucose.

SUBJECTS AND METHODS

Subjects were chosen from the obstetrical in-patient and out-patient services of the New York Hospital. Only

¹ This study was aided by a grant from the John and Mary R. Markle Foundation.

women whose course and past history were considered normal were chosen. Individuals with histories of previous glycosuria, or current diabetes, hypertension, kidney or liver disease were eliminated. The tests were performed on subjects at various stages of pregnancy. Subjects for control study were chosen from gynecological in-patients. They were all within the child-bearing age. Only those with benign conditions were selected.

There were 20 in the pregnant group. Their ages ranged from 17 to 43 years with an average age of 28.9 years. There were five in the fourth, three in the fifth, two in the sixth, three in the seventh, three in the eighth, one in the ninth, and three in the tenth lunar month of pregnancy. There were 11 in the non-pregnant group. Their ages ranged from 20 to 38 years with an average age of 28.9 years.

All subjects were in the post-absorptive state. Some were encouraged to drink 600 to 800 ml. of water before the test was started in order to promote urine flow. A No. 18 Foley indwelling catheter with a 5-ml. bag was placed in the bladder and the bladder emptied. A blood sample was withdrawn. Then 50 ml. of 50 per cent glucose were injected intravenously through a No. 18 needle in 45 to 60 seconds. Blood samples were then drawn at five, 15, 30, 60 and 120 minutes after the injection. Urine specimens were drawn at 15, 30, 60 and 120 minutes after the injection, the bladder being completely emptied but not washed out at the time of each collection.

Protein-free filtrates of the blood samples were made by the Folin-Wu method (10). Blood sugars were determined on these filtrates by the Benedict method (11). Quantitative urine sugars were determined on appropriately diluted urine specimens by the same method. The optical densities of the final colored solutions were determined in a Klett-Summerson photometer using filter 42 (12).

RESULTS

The blood sugar values for the pregnant and the control groups are presented in Table I and are illustrated graphically in Figure 1 where the data of Lozner *et al.* are added for comparison. The values obtained by us for the control group parallel closely the values obtained by Lozner *et al.* The values obtained for the pregnant group, however, tend at all times to average slightly, though not significantly, lower than those of the control

groups. At 60 minutes the average blood sugar value and the range of values observed in the pregnant group tend definitely to be lower than the average values observed in the control groups. The difference between the two means equals 3.09 standard errors of the difference between the two means when our data are used, and 2.72 when the data of Lozner *et al.* are used. These figures do not indicate a really significant difference.

The average values, the extreme observed values, and the standard deviation of the pregnant and the nonpregnant groups are summarized in Table II. The standard deviation was calculated in the usual way where the standard deviation

$$\sigma = \sqrt{\frac{\sum d^2}{N-1}}.$$

The amount of glucose excreted in the urine seems to be about the same in both the pregnant and the nonpregnant groups. These values are,

however, nearly twice that reported by Lozner *et al.*

DISCUSSION

From these results it would appear that the pregnant women can tolerate intravenously injected glucose as well as, if not better than, the nonpregnant women. The tendency for the blood sugar levels of the pregnant women to fall toward fasting levels more rapidly, *i.e.*, to have slightly lower blood sugar values at 60 minutes after the injection of the glucose, might indicate a slight increase in the rate of utilization of the glucose, or a greater total volume in which to dilute the glucose or a decreased kidney tubular reabsorptive capacity for glucose (kidney threshold). Or all three of these factors might be operating simultaneously to some extent.

Our present data (the average amount of glucose found in the urine of the pregnant and the

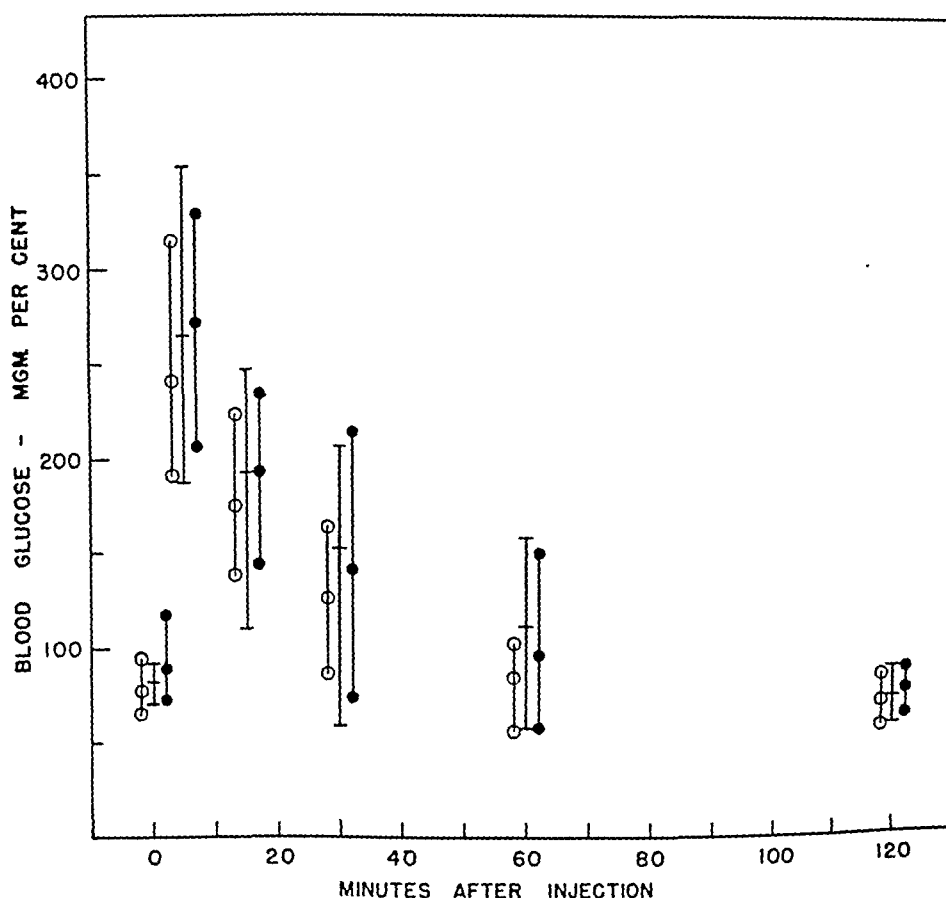


FIG. 1. GRAPH OF RESULTS OF INTRAVENOUS GLUCOSE TOLERANCE TESTS

Open circles indicate data from pregnant women, horizontal lines from normal women and dots from Lozner *et al.* (9). The upper and lower circles (lines or dots) indicate the extremes observed; the central circle the average value. The vertical lines are visual guides. The data from pregnant women are plotted two minutes early and the data from Lozner *et al.* two minutes late.

TABLE I
Distribution of values

	Number	Age	Duration preg. (wks.)	Fasting	5 min.	15 min.	30 min.	60 min.	120 min.
Pregnant group	1	29	29	69	—	152	126	79	59
	2	39	40	65	198	170	130	99	63
	3	23	15	82	202	170	147	92	66
	4	43	22	83	245	171	127	92	73
	5	23	18	90	206	154	93	81	86
	6	21	15	68	231	143	96	56	84
	7	23	39	69	222	149	109	74	63
	8	33	30	79	224	181	137	108	71
	9	37	22	84	257	161	131	97	63
	10	30	32	78	250	213	139	78	75
	11	34	20	94	243	179	137	96	73
	12	17	26	75	286	210	156	103	78
	13	25	34	70	220	163	133	96	70
	14	36	15	79	315	138	99	75	74
	15	21	26	69	230	160	106	83	74
	16	35	37	79	191	167	86	69	70
	17	32	20	79	258	218	165	91	75
	18	24	27	74	315	191	118	62	76
	19	31	13	77	237	179	118	72	68
	20	22	15	81	245	223	164	97	78
Control group	1	30		79	229	174	117	66	73
	2	24		92	230	195	162	147	72
	3	30		83	300	200	130	82	66
	4	22		79	187	138	123	84	69
	5	25		80	273	217	189	134	79
	6	27		84	245	192	159	103	60
	7	20		86	301	195	174	136	78
	8	26		91	354	247	183	107	77
	9	38		70	271	111	58	58	73
	10	29		70	244	211	178	152	90
	11	25		85	282	240	207	160	91

nonpregnant groups) do not indicate a marked decrease in the kidney tubular reabsorptive capacity for glucose in the pregnant women. It must be admitted, however, that the average value for the amount of glucose found in the urine in the pregnant group is about twice the amount observed by

Lozner *et al.* Since, however, we obtained a value of the same order of magnitude in our pregnant and nonpregnant groups, the discrepancy may be due to the use of different methods for the determination of the urine sugar. The actual determination of the maximal kidney tubular reabsorptive capacity for glucose in the pregnant women will answer this question.

Since the glucose tolerance test becomes more like that observed in the nonpregnant women in the early puerperium, one factor operating toward this tendency toward lower values at 60 minutes following the administration of the glucose may be the increased total volume in which the glucose may be distributed. When equilibrium is reestablished essentially normal values obtain. On the other hand, no correlation with the time of pregnancy can be seen if the data are arranged in order of the weeks pregnant. Several different mechanisms may, therefore, operate to produce this tendency.

Intravenously injected glucose seems, then, to be removed from the blood of pregnant women at essentially the same rate as from the blood of nonpregnant women. It becomes difficult now to explain the previously reported tendency toward a decreased tolerance of pregnant women to glucose administered by mouth. This apparent paradox needs further elucidation.

This contradiction in results obtained by the intravenous and the oral glucose tolerance test in pregnancy shows the oral route of administration,

TABLE II
Analysis of data of pregnant and control groups

Pregnant group						Control group					
Minutes after injection	Number of subjects	Glucose in mgm. per 100 cc. blood				Minutes after injection	Number of subjects	Glucose in mgm. per 100 cc. blood			
		Max.	Min.	Aver.	Stand. devia.			Max.	Min.	Aver.	Stand. devia.
0	20	94	65	77	7	0	11	92	70	82	7
5	19	315	191	241	35	5	11	354	187	265	45
15	20	223	138	175	25	15	11	247	111	193	40
30	20	164	86	126	23	30	11	207	58	153	42
60	20	103	56	85	14	60	11	160	58	112	36
120	20	86	59	72	7	120	11	91	60	75	9
Average urine excretion 7.2%						Average urine excretion 6.8%					

with its undesirable and uncontrollable factors in the nonpregnant state, to be less desirable in the pregnant state. Further, there is less objection on the part of the patient to the intravenous administration of 50 per cent glucose than to the ingestion of large quantities of glucose by mouth. In this series the rate of administration of the glucose was as rapid as possible using a No. 18 gauge needle. The only complaint registered by the patients in all instances was a sensation of "warmth" first appearing at the site of the injection and later in the neck. The sensation appeared within 30 seconds of the start of the injection and had completely vanished within 30 seconds of the completion of the injection.

SUMMARY

Intravenous glucose tolerance tests have been performed on 20 pregnant women and on 11 nonpregnant women within the childbearing age. The intravenously injected glucose disappears from the blood of the pregnant women at essentially the same rate as from the blood of nonpregnant women. There is a tendency for the blood sugar of the pregnant women to return to fasting levels more rapidly than of the nonpregnant individuals. These results are discussed. It is concluded that the tolerance for intravenously injected glucose is essentially the same in the pregnant women as in the nonpregnant women of childbearing age.

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THE GENETICS OF GOUT AND HYPERURICEMIA—AN ANALYSIS OF NINETEEN FAMILIES

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At an earlier period, when less confidence was generally held for the gene theory of inheritance, it was natural to insist that any genotype postulated to explain a given disease or abnormality should be perfectly correlated with this disease. When it became increasingly realized that this supposition was unnecessary, a fruitful method of study became available for those diseases showing only occasional evidence of inheritance. By assuming that a given genotype might cause only a portion of its possessors to develop the trait in question, an irregular pattern of inheritance was often found to be adequately explained on the basis of a single gene hypothesis. However, merely to satisfy the requirements of a formal theory would, in general, have represented but little gain. A more important result was that increased attention was now accorded those individuals who, although lacking the disease or abnormality, were evidently carriers of the genetic factor. As a consequence, several important hereditary diseases are now known to be frequently replaced by detectable sub-clinical manifestations or "carrier states," and these conditions have been recently discussed in extensive reviews (1, 2).

In the field of metabolic disorders, Garrod (3) was among the first to emphasize this point of view, notably with respect to gout. He suggested that this disease would probably be found to be due to a dominant Mendelian factor which expresses itself fairly regularly in hyperuricemia, but only occa-

sionally in gouty arthritis. A number of studies (4-7) have since shown that hyperuricemia without arthritis is indeed a common finding among the relatives of gouty patients. The most extensive studies thus far reported are those of Talbott and of Stecher and Hersh. Talbott (5) investigated 136 relatives of 27 patients and found hyperuricemia (serum uric acid exceeding 6 mgm. per cent) in 27 male and seven female relatives. Unfortunately this excellent body of data has thus far been published only in an incomplete form which does not permit of statistical or genetic analysis. A biochemical study of 30 gouty families has also been reported in abstract form by Stecher and Hersh (7).

The present paper presents an analysis of 19 gouty families studied at the Arthritis Clinic of the University of Michigan Hospital during the years 1938 to 1942. Two of these families have been previously reported by Smyth and Freyberg in a paper (6) which will serve to describe more fully the methods used in the investigation. Although these two families demonstrated a marked hereditary tendency in hyperuricemia, the question was left unsettled as to whether all cases of gout can be encompassed in a single genetic theory. The present analysis was carried out in 1942. The results showed that the hypothesis of a dominant autosomal gene for hyperuricemia will satisfactorily explain the variations in serum urate concentrations found among an unselected series of families, but only after allowance is made for certain other factors influencing the level of serum urate in these relatives.

DESCRIPTION OF DATA

The 19 families which form the basis of this report constitute Kindreds 1109 to 1127 in the files of the University of Michigan Heredity Clinic. In this paper the families are designated by the letters A to S, respectively. The *propositus* ("index case" or original patient) is in each case a male patient showing clinical, biochemical

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and, in most cases, radiological evidence of gouty arthritis. A total of 87 relatives was studied and the data concerning these individuals are incorporated on the pedigree diagrams of Figure 1. In general, the pedigrees show only those individuals whose bloods were tested for serum urate, but a few additional members have been portrayed in order to define relationships between ex-

amined members. The pedigrees are therefore incomplete in some cases in the sense that all brothers and sisters of certain individuals are not shown.

In almost all cases the blood samples were obtained after a fast of eight to 12 hours. The blood was allowed to clot under oil, and uric acid determinations were made on the serum using the indirect method of

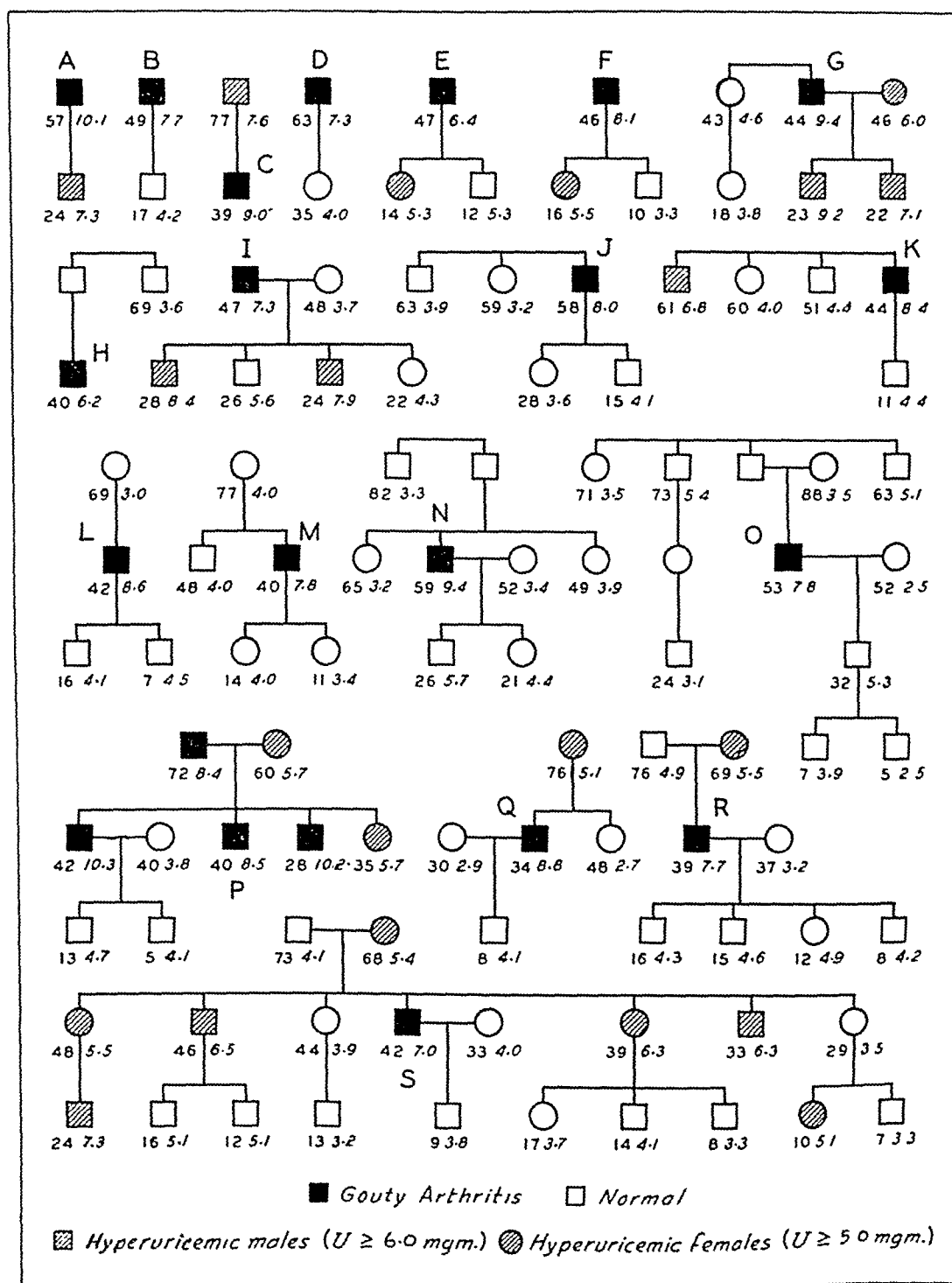


FIG. 1. PEDIGREES OF 19 GOUTY MALE PATIENTS

The 19 families are designated by letters, A-S, placed nearest the proband or original patient in each case. Figures below the pedigree symbols represent age (T) in years, followed by serum urate concentration (U) in mgm. per cent.

Folin (8). The italicized figures beneath the pedigree symbols in Figure 1 indicate the serum urate levels found for each individual, expressed in milligrams uric acid per 100 cc. of serum. In most cases only one determination was obtained for each relative, but a few of the recorded figures represent averages of several determinations.

The two families previously reported (6) are identical with families I and P in this paper. In the latter family, the proband, who is represented by the symbol nearest the letter P in Figure 1, was found to have two gouty brothers and a gouty father. No other relatives belonging to any kindred were known to have a history suggesting gout, and none was found to have tophi. The 19 patients were selected for study solely on the basis of the availability of their relatives, and blood samples were secured on all individuals who were willing to cooperate. It therefore seems likely that the resulting data should provide unbiased estimates of the frequency of hyperuricemia among relatives of any given class.

REGRESSION ANALYSIS

Before examining the data from the point of view of a specific genetic theory, we have analyzed the variation in serum urate levels by means of general statistical methods. This was done in order to ascertain (1) whether there was evidence of genetic variability of any kind, (2) whether age and sex were additional factors influencing the level of serum urate, and (3) whether there was any justification for dividing the relatives into "normal" and "hyperuricemic" groups in the hope that these would correspond to two specific genotypes.

In addition to the serum uric acid concentration (U), Figure 1 also supplies information on the age (T), sex (S), and mode of relationship (R) of each relative to the gouty proband. As a preliminary procedure, we chose to investigate the influence of R , S , and T on U by means of multiple regression statistics (cf. Snedecor [9]). Instead of dividing the data into various sex and relationship classes, we have assigned to each relative a score for sex, $S = 0$ for males and $S = 1$ for females, and have used the coefficient of relationship (10) as a measure (R) of the degree of relationship between the proband and each of his relatives.

It will suffice to explain here that the coefficient of relationship (R) is a measure of the degree of genetic resemblance between any two relatives, and its value varies from $R = 0$ for unrelated individuals, to $R = 1$ for "identical twins." For parents, sibs and children of the proband $R = 0.50$, while for grandparents, aunts, uncles, nieces, nephews and grandchildren $R = 0.25$. All relatives in the present series of pedigrees fall into one or the other of these two degrees of relationship, except for one first cousin once-removed of patient O whose coefficient of relationship to O is $R = 0.0625$. Now, for any autosomal gene present in a gouty patient, the probability that this gene will also be present in a given relative is equal to his or her coefficient of relationship to the patient (10). Therefore, since we shall later wish to test the hypothesis of a dominant autosomal gene for hyper-

uricemia, the use of R is appropriate in the present problem.

The data of Figure 1 may thus be summarized by tabulating for each relative the four variables R , S , T and U . For example, for the uncle of patient H, Figure 1 shows that

$$R = 0.25, S = 0, T = 69 \text{ yr.}, U = 3.6 \text{ mgm. per cent.}$$

The wives of seven probands and of one gouty brother of patient P were also tested, and these unrelated individuals are included in the analysis as "relatives" of degree $R = 0$. Thus, for the wife of patient Q, Figure 1 shows that

$$R = 0, S = 1, T = 30 \text{ yr.}, U = 2.9 \text{ mgm. per cent.}$$

In order to discover whether age (T), sex (S) and degree of genetic relationship (R) significantly affect the uric acid level in relatives of gout patients, we applied the method of multiple regression analysis (9). A regression equation of the usual form,

$$U' = a + bR + cS + dT,$$

was fitted to the data on all 87 relatives. In this equation U' is the predicted uric acid level, a is a basic level of serum urate (the amount to be expected in an unrelated male at birth, i.e., an individual having $R = S = T = 0$), and b , c and d are the "partial regression coefficients," that is, the amounts by which the urate level changes with unit increases in R , S and T , respectively. The derivation of the constants a , b , c and d was carried out by means of the usual methods of regression analysis (9), except that the sums of squares and products of deviations in the four variables were computed from the means of the 19 sets of relatives belonging to the separate pedigrees, rather than from the general mean of all 87 relatives. In this way we obtained the following equation for predicting the uric acid level of a relative from the age, sex and degree of relationship to the gouty patient:

$$U' = 3.5085 + 3.2205R - 0.9443S + 0.011808T \text{ mgm. per cent.}$$

The tests of significance of the deviations of the three regression coefficients from zero were carried out on the corresponding standard partial regression coefficients (9) (Table I). Those for sex and degree of relationship are statistically significant at the 1 per cent probability level, whereas the positive regression of uric acid concentration on age falls short of the 5 per cent level of significance.

TABLE I

Partial regression of U (serum urate level) on:	Regression coefficient, β	Standard error, s_β	$t = \beta/s_\beta$
R (degree of relationship)	+0.37022	± 0.10668	3.470*
S (sex)	-0.34147	± 0.11194	3.051*
T (age)	+0.19079	± 0.11126	1.715

* Highly significant value for 65 degrees of freedom.

Relationship: The coefficient $b = +3.2205$ may be interpreted as meaning that an identical twin ($R=1$) of a gouty patient would be expected to have a uric acid concentration about 3.2 mgm. higher than that of an unrelated person ($R=0$), as judged from the average excesses of $0.5b = 1.6$ mgm. for parents, sibs and children, and $0.25b = 0.8$ mgm. for aunts, nieces, grandchildren, etc. This tendency for the uric acid level to increase with increased relationship to gouty patients may be taken as a general indication of heredity, or, at least, of certain factors operating within kindreds in a manner simulating inheritance.

Sex: The coefficient $c = -0.94433$ shows that females ($S=1$) average about 1 mgm. less uric acid than male relatives ($S=0$) of the same age and degree of relationship to gouty patients. This difference compares favorably with that found by other investigators for unselected adult males and females. We have calculated the means and standard deviations of U from data presented by Jacobson (4), Brøchner-Mortensen (11), and Bulger and Johns (12). Although these workers employed different biochemical procedures, the results (Table II) agree in showing a significantly higher mean uric acid value in males. The variances or standard deviations of U , however, are not significantly different in the two sexes. It is therefore apparent that any genetic theory proposed for hereditary hyperuricemia must take this sex difference into account, and that if relatives of gouty patients are to be classified as "normal" or "hyperuricemic," different critical levels of serum urate will probably have to be adopted for the two sexes. These levels may of necessity be different for various investigators, owing to systematic differences in biochemical techniques.

Age: The coefficient $d = +0.011808$ shows that uric acid levels increase by about 0.01 mgm. per cent per year for the whole of our data, but the increase is not sufficient to establish itself as significantly greater than zero.

TABLE II

Published studies of blood uric acid concentration in unselected adult males and females

Investigator	Number <i>n</i>	Mean \bar{u}	Standard deviation, <i>s</i>
		mgm. %	mgm. %
Brøchner-Mortensen (11)			
Males:	25	7.62	0.917
Females:	25	6.35	1.057
Difference:		1.27*	-0.140
Jacobson (4)			
Males:	63	4.4	1.079
Females:	37	4.0	1.052
Difference:		0.4*	0.027
Bulger & Johns (12)			
Males:	62	4.49	0.974
Females:	41	3.59	1.098
Difference:		0.90*	-0.124

* Highly significant difference ($P < .01$).

The total correlation coefficient for age and serum urate concentration is $+0.222$ for the 48 male relatives and -0.098 for the 39 female relatives, and these values are likewise non-significant. The data therefore fail to establish any definite relationship of serum urate concentration to age as measured by linear regression over the entire age range. However, the scatter diagram for male relatives (Figure 2) shows that none of the 22 boys below 18 years of age had serum urate exceeding 6 mgm. per cent, whereas the 26 older males are equally divided at this level (Table III). The probability of this extreme association in sampling from a population in which hyperuricemia is equally common in the two age groups* is only $35!26!/48!13! = 0.00005$.

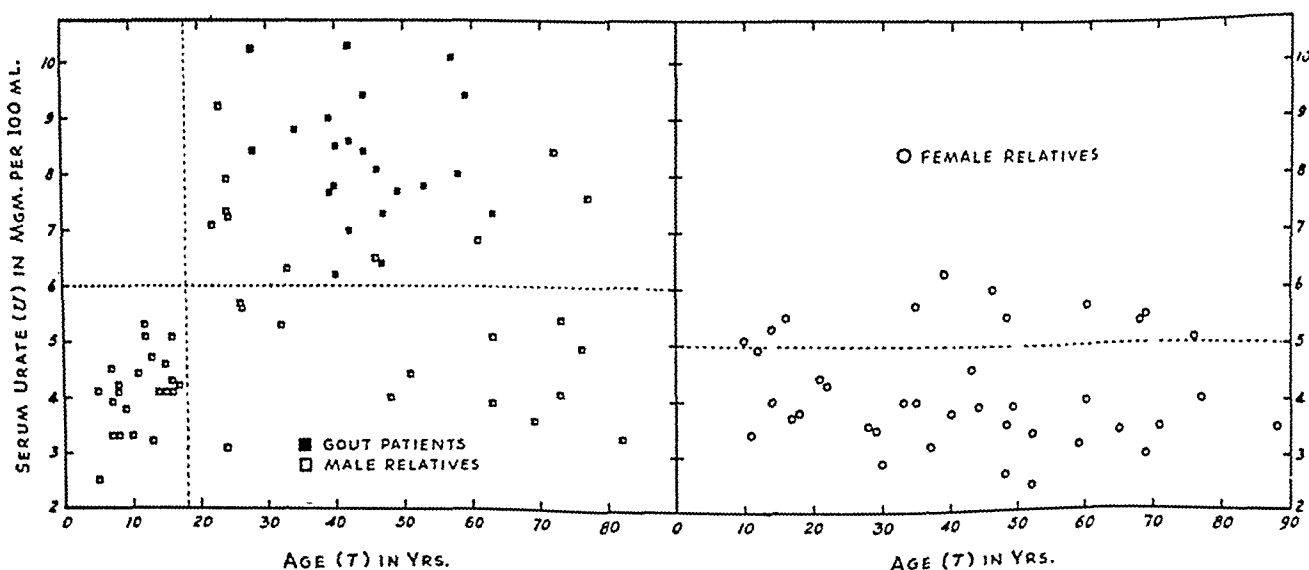


FIG. 2. DISTRIBUTION OF GOUTY PATIENTS AND RELATIVES WITH RESPECT TO AGE AND SERUM

TABLE III

Male relatives	Under 18 yr.	Over 18 yr.
Hyperuricemic ($U > 6$ mgm.)	0	13
Normal ($U < 6$ mgm.)	22	13

We have interpreted this result to mean that the serum urate concentration is probably little, if at all, elevated in young males who possess the genetic factor or factors for hyperuricemia and gout. However, an alternative explanation should be mentioned at this point. It will be seen from Figure 1 that all but four of the 22 males below 18 years of age are sons of gouty or hyperuricemic males, or sons of such sons. The absence of hyperuricemia in this group might therefore suggest that males do not inherit factors for hyperuricemia from their fathers. This would suggest sex-linkage, but the whole of the information available does not support this view, as will be shown in a later section.

ANALYSIS OF VARIANCE

Although sex and degree of relationship to gouty patients have been shown to be significant factors affecting the level of serum urate, the data give evidence of certain kinds of heterogeneity. In Table IV an analysis of

TABLE IV

Analysis of variance for kindreds

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between kindred means	18	88.0338	4.8908*
Within kindreds	68	144.3202	2.1224
Total	86	232.3540	

* Highly significant difference.

variance (9) is set forth for the variations in urate concentrations within and between kindreds. A comparison of the mean squares for these components ($F = 4.8908/2.1224 = 2.3044$) shows that there are significant differences among the means of relatives belonging to different pedigrees. When allowance is made for the fact that these 19 sets of relatives differ in their composition with respect to age, sex, and degree of relationship, we obtain, through the use of regression equations (9), the analysis shown in Table V. The variance among adjusted means of kindreds is now even greater in relation to the residual variation within kindreds, and is again highly significant ($F = 4.8166/1.5376 = 3.1325$).

It therefore appears that serum urate levels in relatives of different gouty patients vary to a degree greater than one would expect by chance if such kindreds were segregating for the same genetic factor producing hyper-

uricemia and gout. Such a result would be anticipated if (1) some of the patients had been incorrectly diagnosed as gouty arthritis, or, what is practically the same, if gout, as diagnosed, were more than a single genetic entity. It would also be explained if (2) environmental factors or modifying genetic factors (in addition to those of age and sex) were unequally distributed among different gouty kindreds. Also the heterogeneity might appear, even in the absence of (1) and (2), if (3) matings between two hyperuricemic individuals occurred in

TABLE V

Analysis of variance for kindreds after adjustment for age, sex and relationship variations

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between adjusted means of kindreds	18	86.6996	4.8166*
Deviations from regression within kindreds	65	99.9431	1.5376
Total	83	186.6427	

* Highly significant difference.

some kindreds but not in all. Some further evidence in favor of (1) and (3) will be discussed in the following section.

Table VI gives the mean uric acid levels for relatives belonging to various age, sex and relationship classes. It is evident that the serum urate level has a higher average value among relatives of degree $R = 0.50$ than among relatives of degree $R = 0.25$, and this is true for both sexes and both age groups. However, the excess is greater in adults than in children and also much greater in males than in females. These discrepancies are statistically significant, and we may conclude that the genetic factor or factors which lead to an elevation in serum urate have a greater average effect in males than in

TABLE VI

Mean uric acid levels for relatives of various age, sex and relationship classes (Frequency in each class given in parentheses)

Male relatives of degree:	Under 18 yr.	Over 18 yr.	All ages
$R = .0625$	— (0)	3.10 (1)	3.10 (1)
$R = .25$	3.93 (10)	4.94 (5)	4.27 (15)
$R = .50$	4.24 (12)	6.70 (20)	5.78 (32)
All males	4.10 (22)	6.22 (26)	5.25 (48)
Female relatives of degree:	Under 18 yr.	Over 18 yr.	All ages
$R = 0$	— (0)	3.69 (8)	3.69 (8)
$R = .25$	4.40 (2)	3.65 (2)	4.02 (4)
$R = .50$	4.62 (5)	4.32 (22)	4.37 (27)
All females	4.56 (7)	4.12 (32)	4.20 (39)

females. The classification of relatives into normal and hyperuricemic groups should therefore prove to be a more difficult task in the case of females.

HYPOTHESIS OF A DOMINANT GENE

So far our analysis has shown merely that some genetic factors in addition to sex may be assumed to account for variations in serum uric acid levels among relatives of gouty patients. Whether it would be profitable to carry this analysis further, by postulating a single gene for hyperuricemia, depends largely on the form of the distribution of urate levels found among the 87 relatives. For example, if instead of blood uric acid levels we had recorded the statures of the relatives of 19 exceptionally tall men, we would have anticipated results very similar to those described above. Males, in general, would be taller than females; the statures would increase with increased relationship to the tall men; and older relatives would be found to be taller than younger ones, although the latter fact might escape detection in a regression equation if adequate numbers of the younger relatives had not been recorded. However, the statures would probably be normally distributed about the means for any age and sex group. Hence, any subdivision of the relatives into "tall" and "normal" would be quite arbitrary, and the hereditary component in stature would be more readily explained on the basis of an inter-play of a large number of genetic factors.

On the other hand, if most of the variability in serum urate levels were determined by a single gene for hyperuricemia, we should expect a bimodal frequency distribution of these levels provided that the separation between the normal and abnormal genotypes were not obscured by relatively large non-genetic variations. Now, it is known that the fasting serum uric acid level is far from a fixed individual characteristic, even at a constant age. Various environmental factors, in addition to technical errors, are thought to be effective in producing marked fluctuations in serum urate determinations when tests are made in single subjects over short intervals of time.

Despite these important individual variations which might be expected to account for the misclassification of some of the relatives, the frequency diagrams of Figure 3 do suggest a bimodal distribution of *U* among relatives of both sexes, and we

have considered it of interest to classify the relatives on the basis of the minimum points in these distributions, *viz.*, 6.0 mgm. per cent in males and 5.0 mgm. per cent in females. The former level agrees with the criterion for pathological hyperuricemia commonly adopted by workers using the Folin procedures. It also agrees with the lower limit found in most cases of gouty arthritis, as is shown by our own data (Figure 3). The lowering of the critical level to 5.0 mgm. per cent in females is suggested by the frequency diagram for females, and this accords with the information cited above from general chemical studies (Table II), namely, that the distributions for the two sexes have approximately equal variances with a mean difference of about 1.0 mgm. per cent.

Using these critical levels 10 of the 48 male relatives and 11 of the 39 females are classed as hyperuricemic. These individuals are shown in Figure 1 by shaded squares and circles, and it is seen that the distribution of hyperuricemic individuals in the 19 pedigrees conforms rather closely with what one might expect if such kindreds were segregating for a dominant autosomal gene for hyperuricemia.

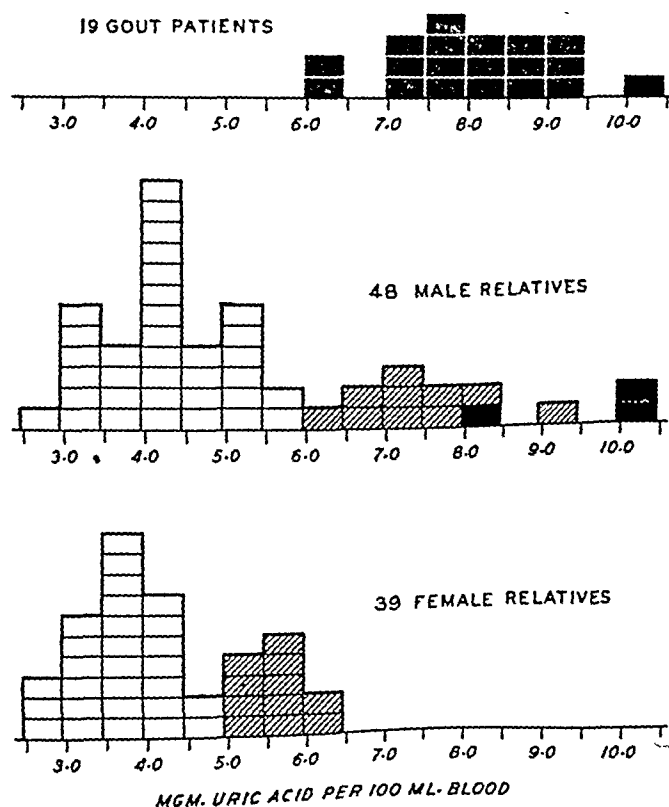


FIG. 3. DISTRIBUTION OF SERUM URATE LEVELS IN GOUTY PATIENTS AND RELATIVES
Each block represents one individual.

TABLE VII

Observed numbers of hyperuricemic (H) and normal (N) children among offspring of various matings

Matings	Sons omitting propositi		Daughters	
	Over 16 yr. H:N	Under 16 yr. H:N	Over 16 yr. H:N	Under 16 yr. H:N
(A) Gouty fathers × hyperuricemic mothers	4:0	—	1:0	—
(B) Gouty or hyperuricemic fathers × normal or untested mothers	3:7	0:12	0:4	2:3
(C) Normal or untested fathers × hyperuricemic mothers	3:0	0:2	2:2	0:1
Total, (B) + (C)	6:7	0:14	2:6	2:4

An enumeration of the children arising from various matings having at least one hyperuricemic parent gives rise to Table VII. In constructing this table, allowance was made for the fact that those sibships which contain the original patient or propositus are biased in favor of hyperuricemic members. The propositus in each case must therefore be omitted. For example, in enumerating the children of a hyperuricemic woman, such as the mother of S, the patient S is omitted and only his sibs are counted, thus giving two hyperuricemic sons, two hyperuricemic daughters, and two normal daughters. Similarly, patient R, whose mother is hyperuricemic, is not counted, because having served as the propositus, it follows that he was necessarily gouty, and therefore, with high probability, hyperuricemic. On the other hand, all of I's children are counted since this sibship was brought to light not through one of the hyperuricemic sons, but through I himself. A fuller explanation of this procedure will be found elsewhere (13, 14).

Discounting those matings in which both parents are hyperuricemic, the remaining sibships contain six hyperuricemic and 21 normal sons. This constitutes a significant departure from the expected 1:1 ratio. However, when sons below 16 years of age are omitted, the ratio becomes six hyperuricemic: seven normal sons. Thus the data concerning male relatives are in agreement with the hypothesis of dominant autosomal inheritance if one assumes that the metabolic change resulting in

hyperuricemia is one which is not manifested in males until about the age of puberty.

Among daughters in the same series of matings (Table VII) four are classed as hyperuricemic, 10 normal. This small number of observations is insufficient to show whether heterozygous females can be regularly distinguished from normal females by their serum urate levels. There is no suggestion here of an age difference in females similar to that found in males. However, it is possible that such may exist, for it is stated by Stecher and Hersh (7) that "hyperuricemia occurs with nearly ten times the normal frequency in brothers, sisters and sons, but not in daughters of gouty patients." ⁴ In Talbott's study (5) males and females were classified alike, using 6.0 mgm. per cent as the criterion for hyperuricemia, and the results show a significantly higher proportion of hyperuricemic males (27 out of 79) than hyperuricemic females (seven out of 57). The serum urate levels of these seven females were also lower than those characteristic of hyperuricemic males, *viz.*, 6.2, 6.2, 6.4, 6.7, 7.0, 7.0, and 10.7 mgm. per cent.

The detection of heterozygous female relatives by means of their urate concentrations is a difficult one, and it may be questioned whether any significant success has been achieved by classifying as hyperuricemic those females having urate levels exceeding 5.0 mgm. per cent. More specifically, we may ask whether the 11 females thus classified are distributed among the 19 pedigrees in a man-

TABLE VIII

Female relatives classified according to their probabilities of possessing the abnormal gene

Female relatives	Probability of possessing abnormal gene as judged from male relatives			
	P=1	P=½	P=¼	P=0
Hyperuricemic ($U > 5.0$ mgm. %) $\frac{11}{19}$	3	7	0	1
Normal ($U < 5.0$ mgm. %) $\frac{8}{19}$	0	19	2	7

⁴ In a full report of their genetic studies now in press (Ann. Int. Med.), Stecher, Hersh, and Soloman report the finding of a significantly increased incidence of hyperuricemic female relatives beyond the age of menopause. No age effect in males was noted, but their series includes only a few relatives under 20 years of age. The possibility that hyperuricemia becomes most evident in males at puberty and in females at menopause is interesting in the light of hormonal studies of Wolfson *et al.* (see below).

ner better suited to the hypothesis of a dominant gene than would be the case for any 11 females taken at random among the 39 females tested. Table VIII shows that this is apparently the case. In this table each tested female is classified according to her probability, P , of possessing the abnormal gene, such probability being inferred from the distribution of hyperuricemic males in her pedigree. For example, on the assumption that all males who are heterozygous have been correctly identified, it is certain (*i.e.*, $P = 1$) that any woman whose son is hyperuricemic and whose husband is not, is herself heterozygous. Of the three females thus judged to be heterozygous (Table VIII, column 2) all occur in the hyperuricemic range. For the eight unrelated wives of the propositi, the probability of possessing the relatively rare gene for hyperuricemia approaches $P = 0$, and only one of these is hyperuricemic. For most of the remaining female relatives the probability of heterozygosity is approximately $P = \frac{1}{2}$, and in this group we find an intermediate ratio: seven hyperuricemic among a total of 26. The proportion $\frac{7}{26}$, however, is significantly less than the expected proportion, $P = \frac{1}{2}$. It is therefore probable that some heterozygous females are not detectable, even when a liberal reduction in the critical level for hyperuricemia is made. Although no case occurs in our material wherein a gouty patient possesses parents both of whom were found to have normal blood uric acid levels, such might therefore be expected, particularly when the hereditary factor was derived from the mother.

Because gouty arthritis occurs much more frequently in males than in females, Talbott (15) has suggested that the responsible genetic factor may be sex-linked. This is made unlikely by the common occurrence of gouty or hyperuricemic sons of gouty male patients, as is seen in the two families illustrated in Talbott's paper (5), as well as in several families in our own series and in the studies of Stecher and Hersh (see above). Under sex-linked inheritance one would also expect higher average urate levels, or a higher incidence of hyperuricemia, among daughters of hyperuricemic males than among daughters of hyperuricemic females. Our data (Table VII) give no suggestion of such a difference.

DISCUSSION

In this paper we have attempted to explain the inheritance of essential hyperuricemia and gout as due to a dominant autosomal (*i.e.*, not sex-linked) gene. Being relatively rare, this gene occurs in a small proportion of males and females who are heterozygous for it; that is, in individuals who have inherited the abnormal factor from only one parent. There is no evidence to suggest what the primary biochemical or developmental effect of this gene may be. We may say only that its presence in the heterozygous condition is detectable in a large percentage of cases by an elevated uric acid level in the blood, and in a much smaller percentage of cases, by clinical symptoms of gout. In discussing the genetics of gouty arthritis and hyperuricemia, care must be taken to distinguish these two states, and our discussion will first deal with hyperuricemia *per se*.

The proportions of hyperuricemic relatives to be expected if heterozygotes were invariably affected are approached when one makes allowances for the factors of age and sex. Reasonably good agreement is obtained in our material by lowering the critical level for hyperuricemia from 6.0 mgm. per cent in males to 5.0 mgm. per cent in females, and by discarding all male children under 16 years of age. The first of these corrections seems to be justified by a well-established difference in the serum urate levels of normal adult males and females, although the deduction of a full milligram may prove to be excessive. The second correction is based wholly on our own data. Classifying males and females separately there still appears to be a deficiency of hyperuricemic relatives, but this is conspicuous only among the relatives making up the final generations in the pedigrees (Figure 1). The deficiency is no longer apparent among the males when children under 16 years of age are excluded. Such a result is commonly observed in pedigrees of retinitis pigmentosa, muscular dystrophy, and numerous other dominantly inherited diseases that have a late age of onset of symptoms.

Concerning the age factor Talbott (5) states: "It is believed that this [hyperuricemia] is a manifestation of a familial tendency. If this assumption is correct, it is probable that an increased concentration is present at birth or shortly thereafter, although the youngest age observed by us was 14."

Only three of the 34 relatives classed as hyperuricemic in Talbott's study were under 18 years of age, these being males aged 14 yr., 17 yr. and 17 yr., having urate levels of 6.4, 6.6 and 7.0 mgm. per cent, respectively. It therefore seems likely that Talbott's data would give evidence of an age effect in males similar to that observed in our material. The finding that marked hyperuricemia is rare before puberty does not, of course, exclude the possibility that a less pronounced elevation may be present, in which case a genetic classification of children might still be feasible. Statistical studies of urate levels in normal subjects of all ages are thus greatly needed for the further study of the genetic carriers in this disease.

Of the 24 hyperuricemic relatives in our study only three were found to have gouty arthritis, and all of these were members of the somewhat unusual family P. Obviously such data throw little or no light on the question as to what factors, in addition to hyperuricemia, may be necessary for the production of clinical gout. However, it would seem worthwhile to point out that the two chief peculiarities which have long been known regarding gout—its predilection for males and its occurrence in middle life—are strikingly paralleled by the facts regarding urate levels of the genetic carriers. If, given the presence of other necessary conditions, the probability of gouty symptoms increases with the absolute level of hyperuricemia reached and with the duration of this condition, then at least a partial explanation would seem to be offered by the differences in the serum urate levels themselves. Thus, the relative infrequency of gouty arthritis in women might be attributed in part to the fact that the abnormal gene, although occurring as frequently in females as in males, has a smaller average effect in elevating the urate level in this sex. This, added to the lower normal urate level in females, results in a much less pronounced degree of hyperuricemia in most of the female carriers. Similarly, the fact that gouty arthritis is principally a disease of middle age is enforced by the finding that marked hyperuricemia is apparently not reached in affected males until about the age of puberty. These "explanations" do not, of course, exclude the existence of other factors which may be important in the production of gouty symptoms and which are wholly unrelated to changes in

the urate concentration of the blood. The fact that other provocative factors are necessary for gouty arthritis is argued by most workers in this field. In this connection, a recent report of Wolfson and co-workers (16) is of interest. These authors found a markedly reduced excretion of 17-ketosteroids in patients with gout, but not in patients having non-gouty hyperuricemia.

We may briefly mention one complication which arises in connection with the theory of inheritance which we have proposed. If we assume that a single dominant gene is necessary for hyperuricemia and gout, it is evident that this gene must be considerably more frequent in the general population than one might suspect from the incidence of gouty arthritis. In our study only three gouty relatives were found among 24 relatives classed as hyperuricemic. In Talbott's study three were found among 34 hyperuricemic relatives. The sex and age distributions of the relatives in these two studies do not differ markedly from those of the general population. Combining the two series, we may take 6/58 or 1/10 as an estimate of the proportion of heterozygotes of all ages who, at any given time, will manifest clinical gout. Among patients visiting arthritis clinics, our own experience agrees with that of other workers in showing that about 4 per cent are victims of gout. Assuming that gout occurs with the same frequency among that fraction of civilians (*viz.*, 2.2 per cent) who are found in health surveys (17) to have histories of "chronic arthritis and rheumatism," we would judge the incidence of gout to be 0.04×0.022 or 88 per 100,000. The frequency of all persons carrying the gene for hereditary hyperuricemia would thus be estimated to be 10×0.00088 or 0.88 per cent. In this calculation the figure 0.00088 is probably too large, since arthritis clinic patients may represent a selected group with respect to gout. On the other hand, the factor 10, taken as the ratio of total carriers to gouty persons, is probably too small, since a considerable number of asymptomatic carriers (especially young males, and females in Talbott's data) have not been detected as hyperuricemic. The two errors may therefore tend to compensate one another in the product.

Taking 0.88 per cent as the frequency of heterozygotes, we should expect matings between two heterozygous individuals to occur with a frequency

of $(0.0088)^2$ or about 8 per 100,000, and the frequency of homozygotes would be one-quarter of this value, or about 2 per 100,000. Now, it is known that certain so-called dominant genes in man are not completely dominant, but produce a more severe abnormality in the homozygous state (1, 2), and it is suspected that such may be the case for many pathological genes. If it were true of the gene for hyperuricemia, and if homozygotes were invariably gouty at an early age, while heterozygotes were only occasionally ($\frac{1}{10}$) afflicted, we should expect these two forms of gout to occur in the ratio 2/100,000:88/100,000, or 1 homozygous case to 44 heterozygous cases.

Furthermore, if homozygous gouty patients were more severely affected than heterozygous patients, such persons would be more likely to visit special clinics and serve as the *propositi* for family investigations. Unless the homozygous condition were lethal in early intrauterine life and therefore unobservable, it seems likely that homozygotes should make up a small, but appreciable, proportion of the clinical cases. Such cases might be expected to show earlier and more severe manifestations, as in a patient described by Ludwig, Bennett and Bauer (18). Both parents would be expected to be hyperuricemic, although neither would necessarily have symptomatic gout. As in the case of rare recessive abnormalities, such cases would be expected to arise with greater probability from consanguineous matings. With the estimated frequency of 2 per 100,000, homozygous patients would perhaps be found to have parents related as first cousins in about 5 to 10 per cent of cases (cf. Dahlberg [19]).

It is interesting that patient P in the present series of families has both parents in the hyperuricemic range according to our classification. One or more members of his sibship might therefore be homozygous, and indeed all of these affected brothers showed rather early ages of onset of acute attacks (6). One might also think of modifying environmental or genetic factors in interpreting families G and P. In any event, biochemical tests on both parents in all branches of a family might be expected to throw considerable light on the above-mentioned questions. This is a defect in our investigation which should be remedied as far as possible in future studies.

SUMMARY

Data are presented on 87 relatives of 19 gouty male patients. Hyperuricemia in these families is apparently due to a single autosomal dominant gene, but only a portion of the heterozygotes for this factor develop recognized gouty arthritis. Sex and age are also significant factors affecting the level of serum urate, and these factors must be taken into account in classifying relatives into "normal" and "hyperuricemic" groups. Males who possess the abnormal hereditary factor apparently seldom develop marked hyperuricemia before the age of puberty. When males under 16 years of age are disregarded, the proportions of hyperuricemic male relatives approach those expected on the assumption that heterozygotes are invariably hyperuricemic. By reducing the critical level for hyperuricemia from 6.0 mgm. per cent in males to 5.0 mgm. per cent in females, the proportions of hyperuricemic females still fall somewhat short of the expected values.

The data are consistent with the view that gouty arthritis may develop in an individual of either sex who possesses sufficiently elevated serum urate level for sufficient time, this being less probable in heterozygous females owing to a lower normal level in women and to a lessened effect of the pathological gene in this sex. It is shown that the gene for essential hyperuricemia must be considerably more common than one might suspect from the incidence of clinical gout, and that homozygotes for this gene should therefore be observed occasionally.

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STUDIES ON CARBOHYDRATE METABOLISM IN PATIENTS WITH GASTRIC CANCER. DEFECTIVE HEPATIC GLYCOGENESIS; EFFECTS OF ADRENO-CORTICAL EXTRACT¹

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INTRODUCTION

Number of metabolic dysfunctions exist in patients with gastric cancer and may contribute to the condition which often occurs in these patients of an adequate diet (1-3). Thus there has been demonstrated an intractable hypoproteinemia which persists in the presence of body fluid depletion (4). Evidence also has been obtained of alterations in the water and electrolyte balance of these patients (5). The present report deals with experiments designed to study carbohydrate metabolism in patients with gastric cancer. It was found that glucose administered by stomach tube to patients with gastric cancer is not transformed into hepatic glycogen and that this defective hepatic glycogenesis can be corrected by the administration of adrenal cortical extract.

PLAN OF STUDY

Liver glycogen depletion after a 12-hour fast. The depletion of hepatic glycogen was first measured in liver biopsies obtained at laparotomy from 38 patients with gastric cancer after a 12-hour fast. These findings were controlled by similar studies in 14 patients with gastric and duodenal lesions.

Hepatic glycogenesis from administered dextrose. In a second experiment, nine patients with gastric cancer received 250 gm. of dextrose in water by stomach tube in five doses given at two-hour intervals for ten hours preceding the operation at which time a liver biopsy was obtained for determination of glycogen. This procedure was repeated on 15 patients with benign gastric lesions.

The effect of adrenal cortical extract on hepatic glycogenesis from administered dextrose. Fourteen patients in this study were aided by grants from the National Institutes of Health, National Cancer Institute and the Fellowship Foundation Fund, New York.

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Patients with gastric cancer were given dextrose as outlined above, and, in addition, received 10-30 ml. of Upjohn's aqueous adreno-cortical extract intramuscularly divided into five doses given every two hours during the ten hours preceding operation.

4) *The effect of insulin on hepatic glycogenesis from administered dextrose.* In six patients with gastric cancer, 5-12 units of insulin were given intramuscularly together with each of five doses of 50 gm. of dextrose administered by stomach tube prior to liver biopsy.

5) *Studies on regulation of blood sugar in patients with gastric cancer.* Intravenous glucose tolerance tests were performed on seven normal subjects and on 13 patients with gastric cancer. In three patients with gastric cancer, this was repeated three times at two-hour intervals. Intravenous insulin tolerance tests were done on five normal subjects and on five patients with gastric cancer.

The effect of a 62-hour fast on blood sugar and urinary nitrogen excretion was studied in three normal subjects and in four patients with gastric cancer. This fasting experiment was designed to test the stability of blood sugar concentration under stress as well as to give information on the extent of hepatic gluconeogenesis from endogenous sources.

METHODS

I. Preparation of patients and biopsies

In the ten hours preceding laparotomy, the patients were either fasted completely or received the medication mentioned (*vide supra*). The amount of residual dextrose solution in all cases was aspirated through the stomach tube at the time of operation, and was found negligible in those cases included in this series. In addition to the medications mentioned uniform pre-operative sedation was employed in all cases. Most of the cases were operated on under pentothal-ether preceded in a few cases by local and spinal anesthesia.

Liver biopsies⁵ were taken immediately upon entering the abdominal cavity and consisted of a piece of hepatic tissue weighing approximately 0.5-1.0 gm. taken from the edge of the right lobe of the liver. This tissue was immediately transferred into a tared dish, weighed and placed in 30 per cent potassium hydroxide for glycogen estimation.

⁵ The cooperation of Drs. G. T. Pack and Gordon McNeer and the staff of the Gastric Service of Memorial Hospital is gratefully acknowledged.

II. Determination of liver glycogen; methods of glucose and insulin tolerance tests; other chemical methods

The liver glycogen was determined by the method of Good, Kramer and Somogyi (6). The glucose tolerance tests were performed by the method of Thorn and associates (7). The dietary preparation periods ranged from three to 62 days. Capillary blood was taken for blood sugar determinations by the method of Somogyi, Shaffer-Hartmann (8) after protein precipitation according to Folin-Wu as modified by Van Slyke and Hawkins (9).

One-tenth of a U.S.P. unit of crystalline insulin per kg. of body weight was used in the intravenous tolerance tests (10).

In the balance studies, nitrogen was determined by a Kjeldahl method in diets and urine.

RESULTS

(1) Degree of liver glycogen depletion after a 12-hour fast

The values obtained for liver glycogen in the fasting patients were identical in the control and in the cancer group (Figure 1, Tables I and Ia).

(2) Hepatic glycogenesis from administered dextrose

The concentration of hepatic glycogen in patients with benign gastric and duodenal lesions following

TABLE I
Benign gastric lesions—no treatment

Liver glycogen gm./100 gm.	Hospital Case No.	Age	Sex	Diagnosis
0.4	56612	35	m	jejunal ulcer
0.4	77727	40	m	gastric ulcer
0.5	75594	52	f	gastric ulcer
0.9	79352	57	m	gastric ulcer
1.3	65722	58	m	gastritis
1.4	64874	63	f	gastritis
1.6	76358	69	m	duodenal ulcer
1.8	71505	51	m	duodenal ulcer
2.8	64784	57	f	peptic ulcers
2.8	76509	50	m	gastric ulcer
3.1	78661	18	m	duodenal cyst
3.6	69269	55	m	gastric ulcer
3.6	64791	60	m	gastric ulcer
4.2	79589	40	m	duodenal ulcer

2.0 average value.

the administration of 250 gm. of dextrose ranged from 4.6 gm./100 gm. of tissue to 9.7 gm./100 gm. of tissue and averaged 7.6 gm./100 gm. of tissue (Figure 1, Tables II and IIa).

In contrast to this, the concentration of hepatic glycogen in patients with gastric cancer under the same conditions ranged from 1.1 gm./100 gm. of tissue to 6.0 gm./100 gm. of tissue (Figure 1).

○ BENIGN GASTRIC LESIONS
● GASTRIC CANCER

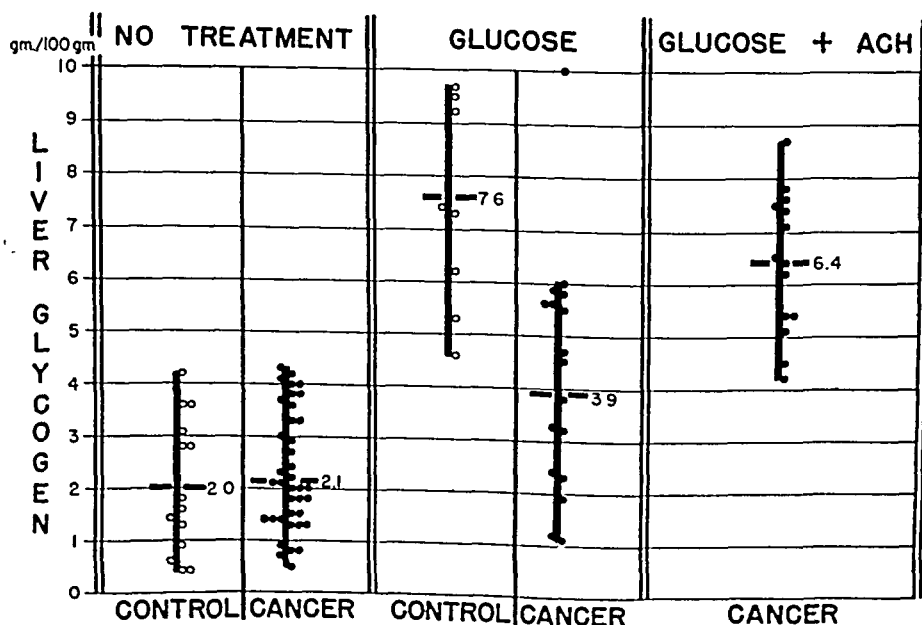


FIG. 1. CONCENTRATION OF LIVER GLYCOGEN IN PATIENTS WITH GASTRIC CANCER AND WITH BENIGN GASTRIC LESIONS

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TABLE Ia
Gastric cancer—no treatment

Liver glycogen gm./100 gm.	Hospital Case No.	Age	Sex	Tumor*	Metastases†	
					Liver	Elsewhere
0.5	66224	53	m	o	—	—
0.7	77377	41	f	o	—	—
0.8	70734	53	f	o	—	—
0.8	68786	46	f	o	—	—
0.9	77532	45	m	o	—	—
1.3	64441	60	m	o	—	—
1.3	75766	64	m	o	—	—
1.3	64910	58	m	o	—	—
1.4	68971	63	m	o	—	—
1.4	77529	35	m	o	—	—
1.4	64216	62	f	o	—	—
1.5	71497	50	m	o	—	—
1.5	65824	57	m	o	—	—
1.8	64409	75	m	o	—	—
1.8	66457	37	f	o	—	—
1.8	64331	59	m	o	—	—
2.0	64866	66	m	o	—	—
2.0	64744	76	m	o	—	—
2.0	65604	65	f	o	—	—
2.1	68997	60	m	o	—	—
2.1	64518	65	f	o	—	—
2.2	79195	58	m	o	—	—
2.3	70528	50	m	o	—	—
2.4	64927	70	f	o	—	—
2.7	65198	54	m	o	—	—
2.9	66545	54	m	o	—	—
3.0	64715	59	m	o	—	—
3.3	70748	62	f	o	—	—
3.3	64821	63	m	o	—	—
3.6	64391	46	m	o	—	—
3.7	68958	62	m	o	—	—
3.8	77655	56	m	o	—	—
3.8	63706	48	m	o	—	—
4.0	69689	43	f	o	—	—
4.0	65407	56	m	o	—	—
4.1	70995	49	f	o	—	—
4.2	65083	46	m	o	—	—
4.3	64618	48	f	o	—	—

2.1 average value.

* Tumor operable = o; tumor inoperable = i.
 † Single or occasional metastasis = +; massive, widespread metastases = +++.

TABLE IIa
Gastric Cancer—glucose

Liver glycogen gm./100 gm.	Hospital Case No.	Age	Sex	Tumor†	Metastases	
					Liver	Elsewhere
1.1	70702*	42	m	o	—	—
1.2	71443	64	m	o	—	—
1.9	70753	69	m	o	—	—
2.3	68530	65	m	o	—	—
2.4	79930	71	m	o	—	—
3.2	81158	69	m	o	—	—
3.3	81115	58	m	o	—	—
3.8	73025	64	f	o	—	—
4.5	65586	62	f	o	—	—
4.7	73406	70	m	o	—	—
5.5	71429	64	m	o	—	—
5.6	71017	53	m	o	—	—
5.8	81560	60	m	o	—	—
5.9	65834	53	m	o	—	—
6.0	79157	58	m	o	—	—

3.9 average value.

* Lymphosarcoma of stomach.
 † Tumor operable = o; tumor inoperable = i.

(There was one exception with a value of 10 gm./100 gm. of tissue in a case of cancer of the esophageal end of the stomach in a woman of 28, the youngest in the entire series.) Excluding this one extreme value the average was 3.9 gm./100 gm. of tissue.

(3) The effect of adrenal cortical extract on hepatic glycogenesis from administered glucose

In 14 patients with gastric cancer who had received adrenal cortical extract together with dextrose, the hepatic concentration of glycogen ranged from 4.2 gm./100 gm. of tissue to 8.7 gm./100 gm. of tissue, with an average value of 6.4 gm./100 gm. of tissue (Figure 1 and Table III).

(4) The effect of insulin on hepatic glycogenesis from administered dextrose

The administration of insulin together with dextrose in six patients with gastric cancer resulted in an insignificant depression of their liver glycogen to an average value of 3.6 gm./100 gm. of tissue (Figure 2).

(5) Studies on the regulation of blood sugar in patients with gastric cancer

There were minor differences between the glucose tolerance curves in patients with gastric cancer.

TABLE II
Benign gastric lesions—glucose

Liver glycogen gm./100 gm.	Number	Age	Sex	Diagnosis
6.6	71428	54	m	duodenal ulcer
6.3	69499	55	m	gastric ulcer
6.2	65622	59	m	duodenal ulcer
6.3	65821	63	f	gastric ulcer
6.3	79862	59	f	abdominal aorta aneurysm
6.3	63450	49	f	duodenal ulcer
6.3	68194	58	m	gastric ulcer
6.3	75119	30	f	gastric polyposis, benign pancreatic cyst

average value.

TABLE III
Gastric cancer—glucose + ACH

Liver glycogen gm./100 gm.	Hospital Case No.	Age	Sex	ACH amount cc.	Tumor†	Metastases	
						Liver	Else-where
4.2	73016	49	m	20	o	—	—
4.5	73033	65	m	30	o	—	+++
5.1	72419	48	f	10	i	—	+++
5.4	72404	66	m	10	o	—	—
5.4	72985	64	m	30	i	—	+
6.2	73548*	59	m	30	o	—	+
6.4	72285	63	m	10	o	—	—
6.5	72615	57	f	20	o	—	—
7.1	72778	69	f	20	o	—	+
7.4	73396	49	m	30	o	—	—
7.5	72227	37	f	20	o	—	—
7.6	73457	70	m	30	o	—	+
7.8	72497	61	f	20	i	—	+++
8.7	72938	68	f	20	i	—	+++

6.4 average value.

* Epidermoid carcinoma of esophagus.

† Tumor operable = o; tumor inoperable = i.

cer and those of patients free of cancer. Thus the average value of the peak of the tolerance curve was 22 mg. per cent higher in the patients with gastric cancer. The slope of the disappearance curve was similar in both groups. The fasting blood sugar levels of patients with gastric cancer were on the average 12 mg. per cent higher than in the controls.

The response to a test dose of insulin was the same in a group of four patients with gastric cancer as in a control group of five normal subjects (Figure 3).

The response to repeated intravenous glucose tolerance tests (every two hours) was essentially

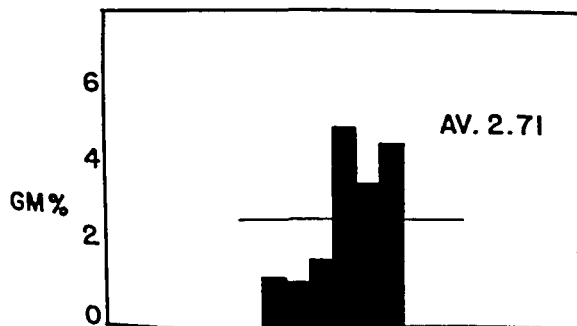


FIG. 2. THE CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF PATIENTS WITH GASTRO-INTESTINAL CANCER AFTER FEEDING OF 250 GM. GLUCOSE AND ADMINISTRATION OF 30-60 U OF INSULIN

EFFECT OF INSULIN (0.1U/kg BW) ON BLOOD SUGAR

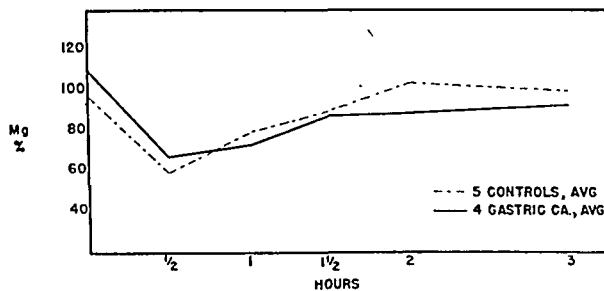


FIG. 3

EXAMPLES OF REPEATED GLUCOSE TOLERANCE TESTS IN A PATIENT WITH GASTRIC CANCER AND IN A NORMAL OF SAME AGE

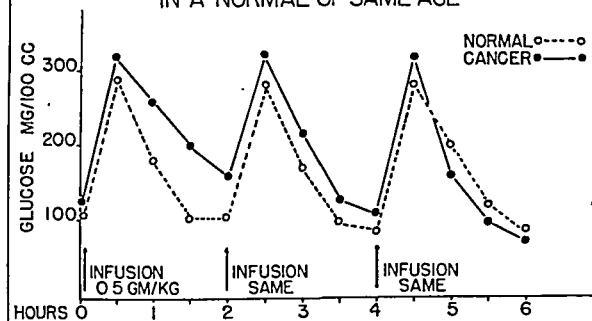


FIG. 4

FASTING EXPERIMENTS

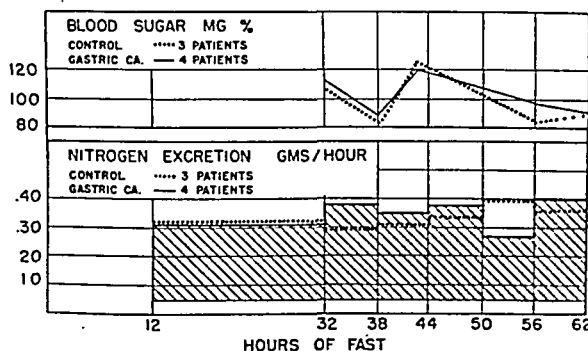


FIG. 5. NITROGEN EXCRETION IN CONTROLS AND GASTRIC CANCER PATIENTS

the same in patients with gastric cancer as in normals (Figure 4).

The blood sugar concentration in patients with gastric cancer during a 62-hour fast closely resembled that found in patients without gastric cancer under similar conditions. The nitrogen excretion in both groups was the same during the fast (Figure 5).

DISCUSSION

These results demonstrate that patients with gastric cancer fail to transform glucose given by stomach tube into hepatic glycogen at a normal rate. The administration of adrenal cortical extract corrects this abnormality. However, the defect is not similar to that prevailing in adrenalectomized animals or in patients with Addison's disease where blood sugar and hepatic glycogen as well as the urinary excretion of nitrogen are lowered by fasting (11, 12). Furthermore, such subjects exhibit marked insulin sensitivity.

The gluconeogenesis from endogenous sources in patients with gastric cancer proceeds normally as manifested by normal fasting concentrations of liver glycogen and by normal behavior of blood sugar and nitrogen excretion during a 62-hour fast (Figure 3). Furthermore, these patients responded normally to the separate intravenous injection of dextrose and insulin as well as to the repeated injection of test doses of dextrose at two-hour intervals. The slight differences in the fasting blood sugar levels and in the peak blood sugar levels during the glucose tolerance tests were satisfactorily explained by the age difference that existed between the control (young) and the cancer group (old) (13). There were no significant differences when individuals of equal age only were compared.

One is thus faced with a dysfunction of carbohydrate metabolism which, while corrected by adrenal hormones, is unlike the disturbance found in adrenalectomized animals. It would appear possible that glucose was poorly absorbed from the gastro-intestinal tract in patients with gastric cancer. However, a similar situation where intestinal malabsorption is ruled out prevails following the intraperitoneal administration of dextrose (14) in mice bearing Sarcoma 180.

The mechanisms of this defective hepatic glycogenesis from administered glucose are as yet poorly understood and call for further studies.

The abnormality is independent of the existence of tumor metastases in the liver or elsewhere and there is no relationship between the size of the total tumor mass and the severity of the disturbance.

The prompt amelioration of this defect by the simultaneous pre-operative administration of glu-

cose and adrenal cortical extract would seem to indicate the use of these measures to prepare patients for major abdominal operations, especially for surgery in gastric cancer.

SUMMARY

1. Patients with gastric cancer fail to transform into hepatic glycogen dextrose that has been given by stomach tube.

2. This metabolic defect is corrected by the injection of adrenal cortical extract.

3. Insulin has no effect on the disturbed hepatic glycogenesis from administered dextrose.

4. This hepatic glycogenesis from endogenous sources in a 12-hour fast is normal and no evidence of disturbed glycogenesis was obtained in a 62-hour fast. The regulation of the peripheral blood sugar and the sensitivity to insulin are normal in patients with gastric cancer.

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THE EFFECT OF SODIUM CHLORIDE DEPLETION ON BLOOD PRESSURE AND TETRAETHYLAMMONIUM CHLORIDE RESPONSE IN HYPERTENSION¹

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A number of investigators have presented evidence to show that a diet poor in sodium may lower the blood pressure of hypertensive patients (1-7). Others have concluded that the blood pressure changes observed in association with sodium restriction are fortuitous (8-13). Recently Perera (6) has shown a small but significant decrease in the early morning blood pressure of a group of hospitalized hypertensive patients in response to a low sodium intake. In his patients the fall in blood pressure was not accompanied by a change in plasma volume, cardiac output, or serum sodium concentration.

In view of the conflicting evidence, it seemed worthwhile to learn first whether more rigid salt deprivation could produce more definite changes in the daily random blood pressure than those previously reported; and second, to define, if possible, the physiologic mechanism concerned in the changes associated with drastic shifts in the sodium balance.

It was thought that information on the latter problem could be obtained by observing the effects of changes in salt balance not only on the random blood pressure, but also on the response of the blood pressure to the tetraethylammonium ion. Since it has been shown that this drug blocks the transmission of impulses by the autonomic ganglia (14, 15), it should be possible to evaluate the relative rôles of neurogenic and humoral tone in the maintenance of the blood pressure (16-20).

MATERIAL AND METHODS

Twelve patients with severe and persistent hypertension were studied to determine the effects of severe sodium

deprivation and then the effects of added sodium upon both daily blood pressure and response to the tetraethylammonium ion. Table I shows the nature of the clinical material. All the patients but one were studied on the medical wards of the Cincinnati General Hospital. No attempt was made to select the patients by type of hypertension. The only essential criterion was a willingness to cooperate in rigid dietary restrictions and to submit to prolonged study in the hospital.

Tetraethylammonium chloride³ (TEAC) was used intravenously in a standard dose of 400 mgm. (4 cc. of solution) and three separate blood pressure values determined: (1) The pre-test blood pressure to which we shall refer as the "random pressure" since we had no fixed hour for testing the patients. (2) The blood pressure during the maximum effect of the TEAC—the "TEAC floor." (3) The fall in blood pressure produced by the drug—the "TEAC response." Because of the rapid and short action of TEAC, determination of random pressure and TEAC floor can be made at daily intervals without affecting the random blood pressure level or the TEAC floor from day to day (22).

The standard procedure for a single evaluation was to take blood pressure readings at minute intervals, with the patient at rest in the supine position for five minutes or longer, until the readings remained consistent for three consecutive minutes. TEAC was then injected rapidly into the antecubital vein of the opposite arm and blood pressure readings determined at 30 to 60 second intervals for six to ten minutes or until the pressure had begun to return toward the starting level. The random blood pressure was taken as the arithmetic mean of three consecutive readings (variation not more than 5 mm. Hg systolic and diastolic) just prior to the injection of the drug. The TEAC floor was recorded as the arithmetic mean of the three lowest pressures reached after the injection of TEAC, taking readings every 30 seconds. The TEAC response was recorded as the difference between the random blood pressure and the TEAC floor. All determinations were done by the same observer. No attempt was made to test the patients in the "basal" state, since we were interested in random pressure levels.

The above studies were carried out during alternate periods of severe salt deprivation and of added salt until

¹ Read in abstract form at the meeting of the American Society for Clinical Investigation, May 3, 1948, Atlantic City, N. J.

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³ Etamon Chloride, furnished by Parke, Davis & Co., Detroit, Michigan, through the courtesy of Dr. E. C. Vonder Heide.

TABLE I
Summary of the clinical material

Name Number	Age Color Sex	Clinical diagnosis	Cardiac status	Renal status		Central nervous system	Fundi (grade*)
				PSP test	BUN (mgm. %)		
Group I							
M. S. 153456	56 Col. Female	Benign essent. hypertens.	Normal function Size: upper normal L. axis deviation	15' 20% 60' 65%	15	Normal	II
E. E. 232262	36 White Female	Benign essent. hypertens.	Recent CHF† L. vent. enlarged Myocard. damage (EKG)	15' 5% 60' 25%	22-35	Acute hyper- tensive enceph- alopathy	II
M. H. 231718	42 Col. Female	Benign essent. hypertens.	Recent CHF "Slightly enlarged" L. bund. br. block	15' 5% 60' 60%	14	Normal	I
G. L. 214414	37 Col. Female	Benign essent. hypertens.	Recent CHF Moderately enlarged "L. vent. strain" (EKG)	15' 10% 60' 60%	14	Previous small thromboses	II
B. C. 4900	58 Col. Female	Benign essent. hypertens.	Recent CHF Markedly enlarged "L. vent. strain" (EKG)	15' 20% 60' 58%	14	Normal	III
Group II							
L. M. 4788	34 Col. Female	Benign essent. hypertens.	Recent CHF Markedly enlarged Myocard. damage	15' 10% 60' 40%	23-30	Chronic hyper- tensive enceph- alopathy	II
I. H. 231469	36 Col. Female	Malignant hypertens.	Function normal Markedly enlarged "L. vent. strain" (EKG)	15' 3% 60' 5%	48-180	Acute hyper- tensive enceph- alopathy	IV
F. R. 11082	44 White Female	Malignant hypertens.	Function normal L. vent. hypertrophy Myocard. damage	15' 2% 60' 10%	18-180	Chronic hyper- tensive enceph- alopathy	IV
J. S. 231439	57 Col. Male	Malignant hypertens.	Recent CHF Markedly enlarged "L. vent. strain" (EKG)	15' 0% 60' 0%	95-160	Clouded (uremia)	IV
F. S. 72244	42 Col. Female	Benign essent. hypertens.	Recent CHF Markedly enlarged	15' 0% 60' 0%	110-180	Clouded (uremia)	II
A. B. 232772	33 Col. Female	Malignant hypertens.	Function normal Moderately enlarged "L. vent. strain" (EKG)	15' 0% 60' 2%	105-160	Clouded (uremia)	IV
F. I. 221329	41 White Male	Malignant hypertens.	Function normal Moderately enlarged Normal EKG	15' 30% 60' 60%	15	Chronic hyper- tensive enceph- alopathy	IV

* Classification of Gifford (21).

† CHF: Congestive heart failure.

a characteristic and reproducible response was obtained for each patient, or until further study was not feasible. A more detailed description of these periods is as follows:

(1) *Control period.* Upon admission to the hospital, or as soon thereafter as the complicating condition would permit, several random pressures and TEAC floors were determined while the patient was on a general house

diet. There were two exceptions: Patient B. C. was not studied in the initial control period because she had been admitted in congestive failure and placed on an effective desalting regimen from the outset. Two subsequent periods of added salt serve as control for her studies, however. Patient F. I. was studied as an ambulatory clinic patient on a regular diet.

(2) "Desalted" period. Following the control period each patient was placed on a low sodium diet which was continued for the duration of the study, except for brief interruptions in the early study of patients G. L. and M. H. In all but one of the cases, the diet was calculated to contain 0.2 to 0.25 Gm. sodium (0.5 Gm. sodium chloride) per day. The one exception, A. B., had very low renal reserve and was given a diet containing 0.9 Gm. sodium. The diets were carefully supervised by one member of the Dietetics Department of the Cincinnati General Hospital. Adequate protein allowance, in the face of such rigid sodium restriction, was made possible by the use of a low sodium milk.⁴ In order to achieve an even greater degree of sodium depletion, mercurhydrin was administered to eight patients in doses of 2 cc. intramuscularly from one to three times per week during

⁴ Lonalac, generously furnished by Meade Johnson & Company, Evansville, Indiana.

the desalting periods. Such a regimen of sodium deprivation was continued for periods of seven to 21 days. The random pressure and TEAC floor were determined two to 19 times while the patient was on this regimen.

(3) "Salted" period. In the six patients whose condition permitted further study, the mercurhydrin was discontinued and 2.4 Gm. sodium (in the form of 6 Gm. sodium chloride in enteric coated tablets) was given daily without a change in the diet, for a period of seven to 12 days. Although the salt added to the regimen constituted only about half the average daily salt intake of adults, this procedure seemed desirable since several of the patients had a history of recent congestive failure. Because of the importance of psychologic factors in hypertension, care was taken not to reveal to the patients the significance of the change in the regimen.

In six patients it was possible to study the changes produced by several alternations of the sodium balance,

TABLE II
Salted versus desalted state—Group I
Comparison of the means and standard errors of random blood pressure, TEAC floor and TEAC response

Patient	State	Periods studied	Number of determinations	Random blood pressure	Stand. error	P*	TEAC floor	Stand. error	P*	TEAC response	Stand. error	P*
M. S.	Salt	3	15	mm. Hg 212.5	mm. Hg ±4.7	.01	mm. Hg 155.4	mm. Hg ±4.7	.001	mm. Hg 57.1	mm. Hg ±5.3	.001
				116.3	±3.1		89.4	±3.0		26.9	±2.9	
	Desalt	3	37	196.3	±3.3	.7	108.3	±5.3	.001	88.0	±6.7	.001
				114.8	±2.7		67.4	±3.2		47.4	±3.6	
E. E.	Salt	4	20	200.0	±7.5	.001	156.9	±7.0	.001	43.1	±6.5	.01
				104.6	±3.8		84.5	±3.2		20.1	±2.6	
	Desalt	3	27	174.4	±4.8	.1	106.0	±5.2	.001	68.0	±5.8	.001
				97.4	±2.2		64.4	±2.8		33.0	±3.3	
M. H.	Salt	2	11	175.5	±3.2	.01	135.6	±4.3	.001	39.9	±5.3	.001
				105.9	±2.0		79.1	±2.6		26.8	±3.1	
	Desalt	2	13	163.8	±2.9	.6	89.7	±5.0	.001	74.1	±6.4	.01
				103.2	±4.7		58.1	±4.6		45.1	±4.5	
G. L.	Salt	2	13	232.2	±4.1	.001	196.7	±4.6	.001	35.5	±5.1	.2
				131.3	±2.4		123.6	±2.5		7.6	±3.2	
	Desalt	2	17	198.0	±4.9	.3	152.4	±5.2	.001	45.6	±5.4	.05
				126.4	±3.7		109.0	±3.9		17.4	±4.0	
B. C.	Salt	2	17	228.0	±3.2	.1	174.8	±4.4	.001	43.2	±5.4	.001
				107.5	±2.4		91.3	±2.1		16.2	±1.8	
	Desalt	3	34	220.0	±3.5	.3	134.3	±4.3	.001	85.7	±5.2	.001
				104.3	±2.0		74.3	±2.2		30.0	±2.3	

* Probability that the observed change could have been due to chance alone. Values of P of 0.05 or less are usually considered significant.

i.e., several "salted" and "desalted" periods. This afforded an opportunity to observe the consistency of the response of the patients to repeated changes in the regimen.

In nine patients random pressures and TEAC floors were determined at intervals varying from one to three days. They were observed from 14 to 63 days. In the remaining three patients, the blood pressures and TEAC floors were determined less frequently. A total of 268 determinations of the blood pressures and TEAC floors were made in the 12 patients.

Serum sodium levels were determined in seven patients at the height of the salting and desalting regimens without regard for alterations in the random blood pressure and TEAC floor. The uranyl-zinc acetate gravimetric method (23) was used for these determinations. The blood samples were drawn under oil and the serum separated from the cells within two hours.

The weight of the patients was recorded as often as practical. All patients were observed closely for signs and symptoms of salt deprivation and of dehydration.

In three patients the cardiac output determinations were made both in a salted and a desalted period by means of the ballistocardiograph. Repeated tracings were taken during routine determination of the random blood pressure and TEAC floor.

RESULTS

The results will be considered in two groups divided on the basis of alterability of blood pressure and/or TEAC floor by sodium deprivation.

Group I. Five patients consistently showed a fall in random blood pressure and/or TEAC floor during desalted periods as compared with salted periods. All of these patients had severe benign essential hypertension and only moderately impaired renal function. They were studied a total of 204 times with TEAC, through 13 salted and control periods and 13 desalted periods (Table II).

Figure 1 shows the alterations that occurred in patient E. E. during 61 days of study. The random blood pressure fell during each period of sodium deprivation and rose during each period of added salt. The change in the level of the TEAC floor was in the same direction as that of the random pressure but the magnitude of the change was greater. The response to TEAC was greater during desalted than during salted periods.

Figure 2 shows a 59-day study of patient B. C., whose renal function was good. The level of the random blood pressure was not affected by the changes in the salt balance. The TEAC floor consistently fell in desalted periods and rose in salted periods. The magnitude of the TEAC response was greater in desalted than in salted periods. The serum sodium values showed no consistent changes in this patient.

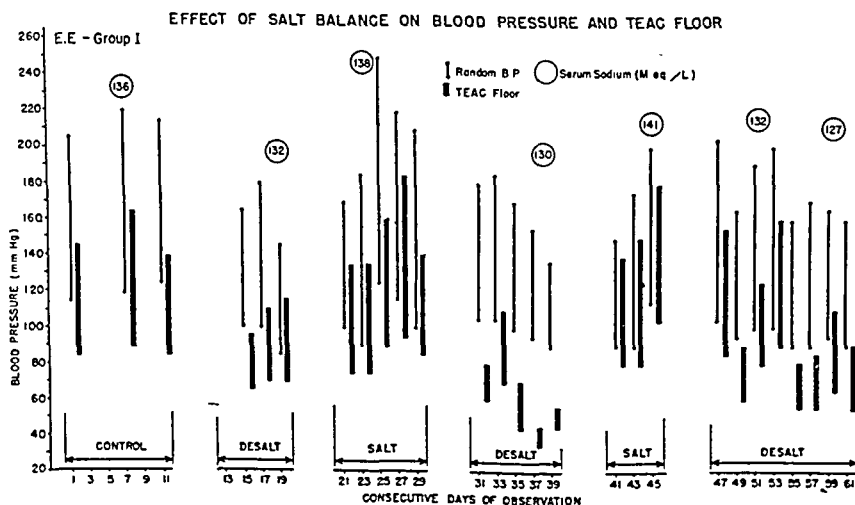


FIG. 1. ILLUSTRATION OF THE RANDOM BLOOD PRESSURE AND TEAC FLOOR CHANGES IN A PATIENT OF GROUP I

The thin vertical lines connect systolic and diastolic values for random pressures and the heavy lines for the TEAC floors. The TEAC response is the difference between the random pressure for a given day and the corresponding TEAC floor. The encircled figures indicate the serum sodium values in Milli-equivalents per liter. To avoid crowding only tests done on odd days are recorded. The breaks in the baseline do not represent lapses of time but divide the study into periods.

EFFECT OF SALT BALANCE ON BLOOD PRESSURE AND TEAC FLOOR

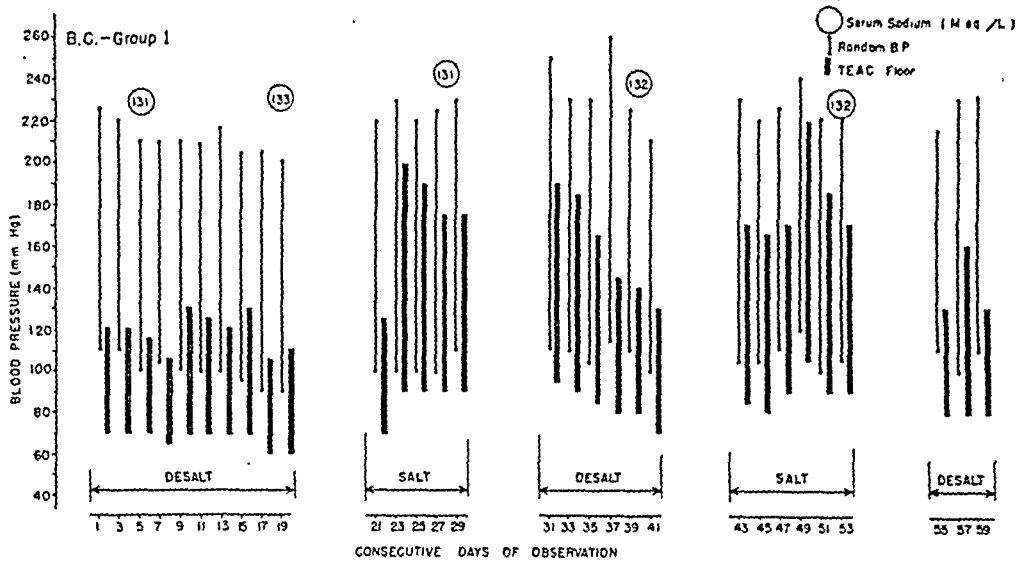


FIG. 2. IN THIS PATIENT THE TEAC FLOORS SHOWED SIGNIFICANT LOWERING AND THE TEAC RESPONSES SIGNIFICANT INCREASES WITH SODIUM DEPRIVATION, BUT THE RANDOM PRESSURES WERE RELATIVELY CONSTANT

The symbols are the same as in Figure 1. The serum sodium changes were neither great nor consistent in this patient.

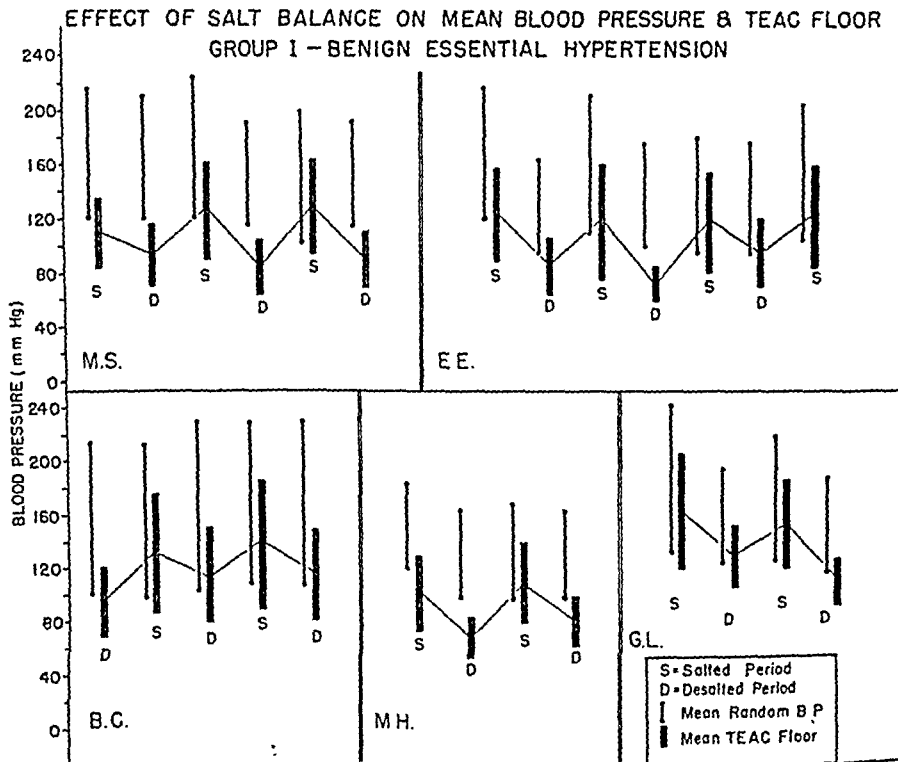


FIG. 3. MEAN RANDOM PRESSURES AND TEAC FLOORS BY PERIODS FOR EACH PATIENT OF GROUP I

The random pressures showed but little change, while the TEAC floors were consistently lower in desalted periods. This illustrates the number of times the changes were reproduced in each patient. The horizontal lines connect the midpoints of the TEAC floors for emphasis.

Figure 3 shows the mean values of the random blood pressure and TEAC floor in all salted and desalted periods for each of the five patients of Group I. All five patients showed a consistent fall in the TEAC floor during desalted periods and a rise during salted periods. This figure also il-

TABLE III

*Salted versus desalted state—Group II**Comparison of the means and standard errors of random blood pressure, TEAC floor and TEAC response*

Patient	State	Periods studied	Number of tests	Random blood pressure	P*	TEAC floor	P*	TEAC response	P*
L. M.	Salt	2	13	<i>mm. Hg</i> 206.5± 3.3	.05	<i>mm. Hg</i> 184.5± 3.0	.02	<i>mm. Hg</i> 22.0± 2.6	.9
				132.6± 2.8		106.5± 2.4		26.1± 2.0	
	Desalt	4	13	195.0± 3.9	.7	173.6± 3.3	.02 Neg.†	21.4± 2.5	.01 Neg.†
				131.0± 1.6		115.6± 2.0		15.4± 2.4	
I. H.	Salt	1	3	269.7±10.5	.1	224.4± 9.4	.8	45.3± 8.2	1.0
				177.0± 5.9		156.7± 4.9		20.3± 3.9	
	Desalt	1	7	251.7± 3.3	.6	227.0± 2.3	.6	24.7± 1.3	.7
				172.0± 5.3		153.1± 3.8		18.9±1.0	
F. R.	Salt	1	4	223.5± 4.2	.02 Neg.	168.0± 9.0	.6	55.5±12.0	.4
				145.5± 6.3		108.2± 6.0		37.3± 5.6	
	Desalt	1	4	259.5±10.6	.1	177.8±16.2	.05 Neg.	81.7±20.4	.8
				165.0± 6.1		130.0± 7.6		35.0± 8.9	
J. S.	Salt	1	3	225.0± 5.0	.1	198.3± 6.2	.01 Neg.	26.7± 7.2	.1
				150.3± 8.5		144.0± 6.4		6.3± 3.4	
	Desalt	1	8	235.6± 3.1	.8	223.4± 3.6	.3	12.2± 4.2	.3
				153.5± 4.8		152.5± 4.0		1.0± 2.7	
F. S.	Salt	1	1	175.0±10.1	.4	160.0±11.4	.6	15.0±12.6	.9
				115.0± 8.7		92.0± 7.6		23.0± 6.5	
	Desalt	1	2	162.5± 2.5	.7	150.0± 5.6	.5	12.5± 7.5	.3
				110.0± 5.0		101.5± 4.3		8.5± 3.5	
A. B.	Salt	1	1	240.0±10.1	.1	220.0±11.4	.2	20.0±12.6	.5
				140.0± 8.7		125.0± 7.8		15.0± 6.5	
	Desalt	1	3	267.7± 8.9	.9	245.1± 7.6	.7	31.6± 5.9	.8
				141.7± 9.3		129.0± 7.1		12.7± 3.9	
F. I.	Salt	1	3	246.3± 8.8	.9	165.0±17.2	.7	81.3±22.6	.7
				171.3± 7.2		118.6±13.6		42.7±17.7	
	Desalt	1	1	245.0±13.4	.7	178.0±15.6	.8	67.0±17.0	.7
				158.0±17.0		125.0±11.6		33.0± 9.1	

* Probability that the observed change could have been due to chance alone. Values of P of 0.05 or less are usually considered significant.

† Neg. indicates that the change was in the opposite direction from the expected change on the basis of the significant changes in Group I.

illustrates the number of times these observations were repeated in each patient and the reproducibility of the responses. Table II shows a comparison of the mean random blood pressure, TEAC floor, and TEAC response, in the salted and desalted periods. In all instances a significant shift occurred in both the systolic and diastolic elements of the TEAC floor. Four of the five patients showed a significant change in the random systolic blood pressure, while none showed a significant alteration of the random diastolic pressure. The variations in the TEAC responses were significant except for the systolic component in patient G. L.

Serum sodium determinations were done at representative times throughout the study of four patients of this group. These values are shown in Table IV. In patient E. E. (Figure 1), whose renal function was moderately impaired, the serum sodium concentration was consistently lower in the desalted than in salted periods. The initial sodium level was also moderately low. During the study this patient manifested clinical evidence

of hypochloremia only once showing weakness, nocturnal delirium and abdominal cramps for three days. This occurred during the second desalted period when her TEAC floor was at its lowest level (Figure 1). At this time the serum sodium level was 130.0 Milli-equivalents per liter, but this was not the lowest level that it reached. The random pressure was about the same as in other desalted periods when the patient showed no symptoms of hypochloremia. In other patients, also, there was no correlation between clinical condition, blood pressure levels and serum sodium concentrations.

All patients lost weight throughout the period of study. A large part of this loss was due to lack of appetite for saltless food. There was in general a loss of 3 to 5 pounds with each period of desalting and a regaining of 2 to 3 pounds with periods of added salt.

It was possible to compute the change in cardiac output produced by TEAC in salted vs. desalted periods in two of the three patients so studied. In Figure 4 the changes in cardiac output and blood pressure observed in patient M. S. are charted. The resting output, before the injection of the drug, was not significantly different in the two periods. It can be seen in this patient that a slight increase in cardiac output occurred after TEAC under both conditions. The increase was, if anything, slightly greater in the desalted period. The change in the peripheral resistance produced by TEAC was significantly greater in the desalted state. Similar results were obtained in patient E. E.

Group II. No significant changes occurred in random blood pressure. TEAC floor, or TEAC response in the seven patients of this group. These patients were more seriously ill than those of Group I, all having malignant hypertension, renal failure with uremia, or both. The typical pattern of response in the patients of this group is illustrated in Figure 5. Table III shows a comparison of the average random blood pressure, TEAC floor, and TEAC response in salted and desalted periods for each of the seven patients. Table IV shows the serum sodium concentrations for three patients of this group. The control levels were low in all three patients, ranging from 129 to 133 M-eq./L. These changes were greater than those in the patients of Group I, although the desalting regimen

TABLE IV
Serum sodium concentrations by periods
(Milli-equivalents per liter)

Name	Control period	First desalt	First salt	Second desalt	Second salt	Third desalt	Third salt
Group I							
M. S.	133.7	134.6* 134.7*	133.4	129.4	136.5	130.7	
E. E.	135.8	132.0	137.7	127.0	141.0	132.2 126.8*	136.9
G. L.		136.2*	138.2 141.8	129.1	135.2 135.8		
B. C.		130.8 133.6*	131.5	131.7	131.8		
Group II							
L. M.				124.3 126.1* 121.3* 116.2*			
F. R.	133.1	111.4	126.5 130.3*				
F. S.	128.8	121.3					
F. I.	133.0	116.6	128.5				

* Where more than one value is reported for a given period the first was done near the middle of the period and the others later in the same period.

EFFECT OF TEAC ON BLOOD PRESSURE & CARDIAC OUTPUT

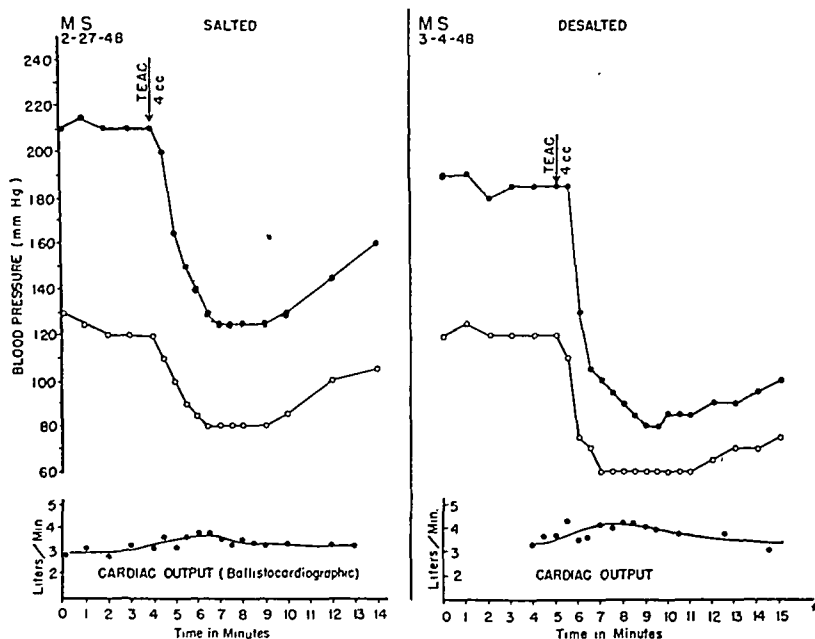


FIG. 4. SIMULTANEOUS CARDIAC OUTPUT (BALLISTOCARDIOGRAPH) AND BLOOD PRESSURE CHANGES IN RESPONSE TO TEAC IN A SALTED PERIOD AND IN A DESALTED PERIOD

The resting cardiac output was about the same and the resting blood pressure was similar. In response to TEAC, however, the fall in pressure was significantly greater in the desalted state. The rise in cardiac output was if anything slightly greater when the patient was desalted.

EFFECT OF SALT BALANCE ON BLOOD PRESSURE AND TEAC FLOOR

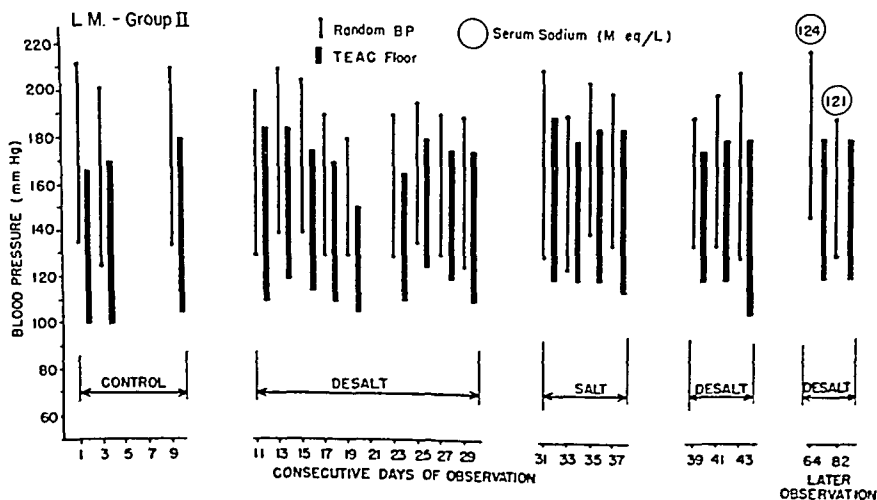


FIG. 5. RECORD OF A PATIENT OF GROUP II TO SHOW THE LACK OF INFLUENCE OF SALT BALANCE UPON THE BLOOD PRESSURE AND TEAC FLOOR OF SOME HYPERTENSIVE PATIENTS

The two observations shown at the far right were done at later times, but are similar to the previous results. The original sodium values for this patient are not determined. Later, however, when the serum sodium concentration was at a very low level the blood pressure and TEAC floor were unchanged.

was less stringent. These data show clearly the lack of effect of alterations of sodium balance on the blood pressure and TEAC floor of the patients of this group.

Figure 6 and Table III show a comparison of blood pressure and TEAC floor during control (salted) and desalted periods in the patients of Group II. Comparison of the mean random blood pressure in salted and desalted periods for any given patient reveals either little change, or a rise in the desalted period. The magnitude of the TEAC response was not increased by desalting in this group.

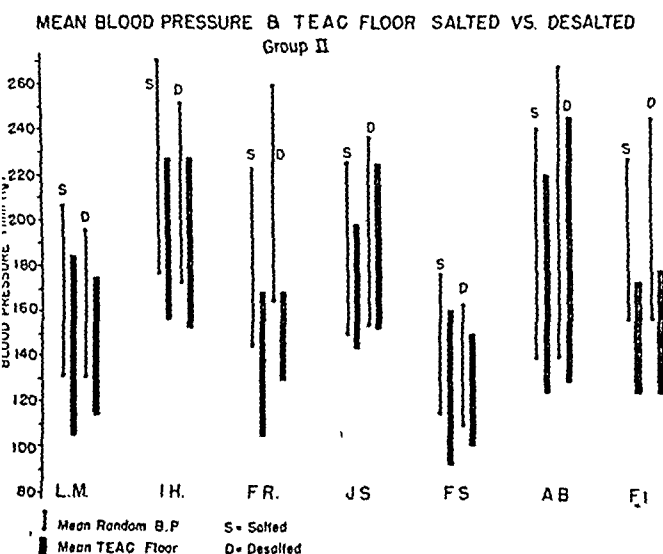


FIG. 6. COMPARISON OF THE MEANS OF RANDOM PRESSURES AND TEAC FLOORS IN CONTROL (SALTED) AND DESALTED PERIODS FOR THE PATIENTS OF GROUP II

There is no fall and in some a rise in both values in association with sodium depletion.

Additional observations on two patients, in whom conditions were not controlled adequately to warrant inclusion in the tables, indicate further the lack of correlation between serum sodium levels and blood pressure in some cases of hypertension. The first of these is patient F. R. (Table I), on whom studies are recorded for the control and one desalted period (Figure 6 and Table III), because from that time there were other complicating factors. While on the salt deprivation regimen the condition of the patient deteriorated and her blood pressure rose. The TEAC floor remained about the same. She lost a great deal of weight, became very dehydrated and stuporous. Her serum sodium level fell from 133.1 to 111.4 M-eq./

L during this period of two weeks of sodium deprivation. At this time she was partially rehydrated by intravenous administration of normal saline solution with considerable improvement. Following this she was given veratrine and magnesium sulfate for 12 hours in doses sufficient to reduce her blood pressure from 270/85 to 160/110 mm. Hg. Concomitantly with this reduction in blood pressure, she lapsed into a coma. Four days later, after her recovery from the coma, the blood pressure and TEAC floor were lower than they had been before the treatment and remained so for four more days. During this time she was clinically greatly improved with the serum sodium level up to 126.5, and five days later the level was at 130.3 M-eq./L. A reinstitution of the sodium restriction regimen was again followed by a rise in the blood pressure and the TEAC floor, while she lost weight and became more dehydrated and uremic. Autopsy showed malignant nephrosclerosis.

The second patient so studied was F. I., whose serum sodium level fell from 133.0 to 116.0 M-eq./L in six weeks on sodium deprivation carried out at home. During this time he became somewhat worse clinically, the random pressure rose and TEAC floor remained unchanged. Addition of salt brought the sodium level up to 128.0 M-eq./L in three weeks. There was a further rise in the random pressure and no change in TEAC floor during this time. Further rehydration and addition of salt in the hospital was associated with a fall in both the random pressure and TEAC floor. This patient had malignant hypertension, but PSP excretion, concentration capacity, and blood urea nitrogen concentration were all normal. His renal defect seemed to be limited to the inability to conserve electrolytes.

DISCUSSION

A relationship between the metabolism of sodium chloride and essential hypertension was suggested many years ago by clinical studies (1, 2, 7). This view has recently been given support experimentally (24-26) and clinically (3-6, 27). The possible role of the adrenal cortex in this relationship was suggested by the action of desoxycorticosterone acetate plus an excessive salt intake in producing malignant hypertension in experimental animals (26). In man an increase of blood pressure has been produced by similar means (28, 29).

Perera (6) was able to show the influence of sodium restriction on the early morning blood pressure of patients with uncomplicated essential hypertension. He found that this "resting" pressure was consistently lower when the patient was on a diet containing only 0.2 Gm. sodium and returned to control levels when salt was added to the regimen. There was no effect, however, on the pressures taken at random throughout the day. The interpretation given was that the basal humoral component in the blood pressure was diminished by sodium deprivation, but that it was compensated by an increase in neurogenic tone as the patient met life through the day. Comprehensive studies failed to reveal any significant change in serum sodium concentration, cardiac output, plasma volume, etc. His patients all had normal cardiac and renal function. Bryant (3), using a similar regimen, found a significant lowering of the pressures of 100 patients followed in the outpatient clinic. On the other hand, the efficacy of salt restriction in hypertension has been doubted by many authors (8-13). Viersma (13) feels that when adequate control periods are studied—up to two to three months—no significant changes in blood pressure follow sodium restriction.

The validity of inferences with regard to the neurogenic contribution to the blood pressure is based on the work of Acheson and Moe (15) and Acheson and Periera (14) who demonstrated experimentally that the tetraethylammonium ion effectively blocks autonomic impulses at the ganglionic synapse. Lyons *et al.* (17-19) have studied the effects of this drug in the human and feel that autonomic ganglion blockade is the chief effect of the drug. Reiser and Ferris (20) have shown that the cold pressor response, which is primarily mediated through the autonomic nervous system, is effectively blocked by this drug in man. Ferris *et al.* (16) feel that the differences in blood pressure response to TEAC, which are noted clinically, are not due to varying degrees of autonomic blockade but can be explained by the variations in the humoral and neurogenic factors operative in different patients with essential hypertension. Similarly, they believe that the relative importance of each of these mechanisms may fluctuate from time to time in the same patient. This would account for the orderly changes in the response to TEAC which they have often observed in re-

peated testing of an individual patient and for the different types of responses which characterize hypertension of different etiologies.

If one assumed, on the basis of the above work, that TEAC achieves an effective blockade of autonomic impulses in the ganglia, the following hypothetical picture of the maintenance of peripheral resistance can be set up: (1) The blood pressure at any time (random or casual blood pressure) reflects the total effect of the humoral and neurogenic factors operating, plus whatever intrinsic tone the vascular bed may possess. (2) The fall in pressure produced by TEAC autonomic block (TEAC response) represents the neurogenic component of that random pressure. (3) The pressure following the drug (TEAC floor) reflects the part played by non-neurogenic factors; *i.e.*, all the humoral agents plus the intrinsic tone in a given random blood pressure.

By taking the above formulation as a working hypothesis, our data could be interpreted as follows: In the five patients with benign essential hypertension, whose renal function was fairly well preserved (Group I), the humoral component of the peripheral resistance (as reflected by the TEAC floor) was altered by changes in the salt balance. These patients had the ability to conserve sodium chloride and thus to maintain a relatively isotonic concentration in the blood even against vigorous attempts at sodium depletion. In the seven patients of Group II, the humoral contribution to the elevated blood pressure (TEAC floor) was not altered by shifting the sodium balance. It is of interest to note that all the latter patients either had severe renal damage in the usual sense, or had malignant hypertension. Viersma has shown that patients with malignant hypertension may have an inability to conserve electrolytes as their only manifestation of renal damage and such inability is commonly observed in chronic nephritis.

The change in the TEAC floor of the patients of Group I was much greater than that in the random blood pressures. This suggests that a compensatory rise in the neurogenic contribution to peripheral resistance may occur when the humoral component diminishes. This is consistent with Perera's findings of an alteration only in the early morning pressure with no effect upon the random pressure throughout the day. This compensation is well illustrated by the patient B. C. (Figure 2) in whom

the random pressure showed no change in the 59 days of study, while the TEAC floor varied significantly with shifts in the sodium balance. In our study the slight changes in random pressures that occurred in some patients may be accounted for by the fact that our desalting regimen was more severe than that used by Perera.

In this interpretation of our data we have assumed that the effect of TEAC is the removal of neurogenic vascular tone by autonomic ganglion blockage. There is a considerable body of evidence to suggest that this assumption is valid, but it has not been conclusively proved in man. The possibility remains that salt deprivation would lower the TEAC floor by altering the ganglionic blocking activity of the drug, instead of altering humoral tone. This possibility does not seem likely in view of present evidence. It appears that the humoral contribution to peripheral resistance may be altered in some patients with benign essential hypertension whose kidneys have the ability to conserve electrolytes for the body. Even in these patients, however, a compensatory rise in the neurogenic contribution to the peripheral resistance may partially or completely offset the decrease of the humoral factor. In other patients whose hypertension is further advanced and whose kidneys have lost the ability to conserve the electrolyte concentration of the body fluids, a further lowering of the body sodium does not affect an apparently large humoral contribution to the peripheral resistance. It is also important to note that even those patients who showed lowering of the random blood pressure and TEAC floor were under closely controlled conditions of a hospital. The results have no bearing on the possible therapeutic effectiveness of salt deprivation in ambulatory hypertensive patients, subject to the usual environmental stresses.

SUMMARY AND CONCLUSIONS

Random blood pressures and tetraethylammonium chloride "floors" have been studied in 12 hypertensive patients subjected to severe changes in sodium balance.

Five patients showed, on desalting, a significant lowering of the TEAC floor—presumably the non-neurogenic or humoral component of the pressure. In four of these the change in random pressure was less striking, apparently because of a

compensatory rise in the neurogenic contribution, as indicated by an increase in the TEAC response in the desalted periods. Serum sodium concentrations were slightly below normal in the control periods, but showed no consistent changes during subsequent salted and desalted periods.

Seven patients showed no change in either random or TEAC floor pressures. The data showed that in these patients a fall of 15 to 20 M.-eq. per liter in the serum sodium concentration was associated with no fall in the random pressure or the TEAC floor. This is in contrast to the patients of Group I in whom a minor change in serum sodium concentration was associated with a striking fall in what seems to be the humoral component of the blood pressure.

In general, the results suggest:

(1) That in some patients with essential hypertension and only moderately impaired renal function, the important change during sodium deprivation may be a lessening of the humoral contribution to the maintenance of an increased peripheral resistance.

(2) That in other patients with malignant hypertension, renal failure, or both, an apparently large humoral component in peripheral resistance may be uninfluenced by an even greater degree of sodium depletion.

ACKNOWLEDGMENT

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THROMBIN FORMATION. I. THE ROLE OF CALCIUM, SERUM AC-GLOBULIN AND TISSUE THROMBOPLASTIN¹

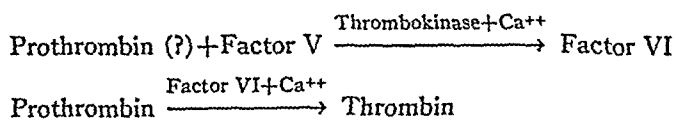
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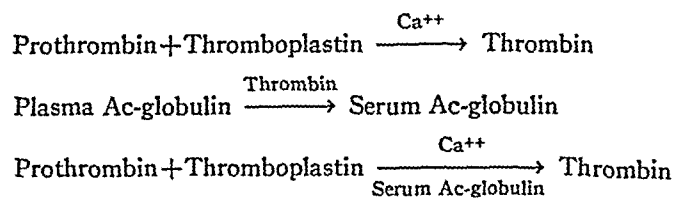
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Recent investigations have shown that thrombin is not formed simply by the interaction of prothrombin and thromboplastin in the presence of calcium ion but that another factor(s) is important and perhaps necessary. Almost simultaneously various workers have described new factors but the different investigational approaches involved have made it difficult to determine if all factors discussed are identical.

Quick (1-3) has described a labile factor disappearing from plasma on storage, but differing from the classical prothrombin. Owren (4-6) reported a new factor, "factor V," which he found diminished in one patient and which he describes as "found in normal plasma, thermolabile, and important to thrombin formation." He expresses its action by the following outline:



Ware, Guest, and Seegers (7, 8) and Ware, Murphy and Seegers (9) have isolated from plasma and from serum two factors, called Ac-globulin, which they believe accelerate the activation of purified prothrombin in the following fashion:



Fantl and Nance (10-12) and Munro and Munro (13) have also reported the presence of clot acceleratory substance(s) in prothrombin free plasma.

We have been fortunate in obtaining specimens

of Dr. Seeger's purified prothrombin and serum Ac-globulin and have designed these experiments to study the formation of thrombin in the presence of these purified reagents. One of us (14) has recently reported extensively on the formation of thrombin from a purified prothrombin product. This work was completed before the identification of Ac-globulin and must now be modified to include this factor.

MATERIALS

Borate buffer (buff.): pH = 7.7 viz. 45 volumes 2.5% H_3BO_3 , 45 volumes 0.5% NaCl, 10 volumes 4% $\text{Na}_2\text{B}_4\text{O}_7 \cdot 12\text{H}_2\text{O}$. Used as a solvent and diluent throughout.

Prothrombin (Pro): 0.1% solution of prothrombin³ prepared from bovine plasma. Lot no. 480414.

Serum accelerator globulin (AcG): 0.1% solution of AcG³ prepared from bovine serum. Lot no. 480205.

Tissue thromboplastin (Tpin): prepared from acetone dried human brain. 0.5 gm. dried brain suspended in 50 cc. buffer, stirred at 37° for 10 minutes, and centrifuged rapidly for 10 minutes. The supernatant, an active thromboplastin containing small amounts of prothrombin and Ac-globulin, was discarded and the precipitate again suspended in 50 cc. buffer, stirred at 37° for 10 minutes and recentrifuged. The opalescent supernatant proves an extremely active thromboplastin almost completely free of prothrombin and Ac-globulin.

Prothrombin, Ac-globulin and thromboplastin were prepared in large lots, divided into daily requirements and stored frozen at -20° C.

Thrombin: Lot no. 480310³ prepared as noted in text.

Fibrinogen (B.F.): 5 gm. of bovine fibrinogen (Armour) were dissolved in 500 cc. of buffer. To this 170 cc. of saturated ammonium sulfate solution were added, the precipitate collected by centrifugation, washed with one-fourth saturated ammonium sulfate, then quickly with cold distilled water to remove traces of ammonium sulfate, and finally redissolved in 500 cc. of buffer. It was divided into daily requirements and stored frozen at -20° C.

METHODS

Thrombic mixtures (T) were prepared from the stated reagents to a volume of 5 cc. with buffer. At stated

³ Obtained through kindness of Dr. W. H. Seegers, Wayne University.

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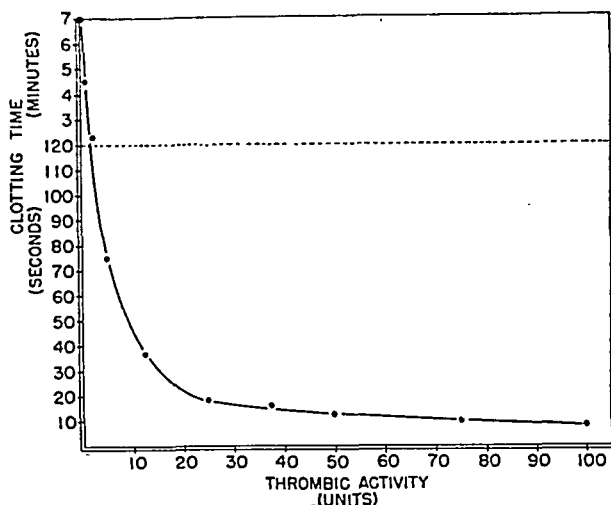


FIG. 1. CLOTTING TIMES OF DILUTIONS OF STANDARD THROMBIC MIXTURE: 100% = 100 UNITS

incubation periods, 0.25 cc. thrombic mixture was added to 0.5 cc. of fibrinogen and clotting time recorded. All procedures were performed at 28° C. unless otherwise stated. Following the first 24-hour incubation period all thrombic mixtures were followed at daily intervals until no evidence of further activation remained and thrombin had started to deteriorate.

To facilitate interpretation of results thrombic activity is presented as a "unit" value. One hundred units (in a volume of 0.25 cc.) of thrombin will clot the standard fibrinogen in 7.5 seconds. This value was chosen as it represents the clotting time obtained when 0.25 cc. of the standard thrombic mixture at full activation is added to 0.5 cc. fibrinogen. The standard thrombic mixture consists of 0.1 cc. of 0.1% prothrombin, 0.1 cc. of 0.1% Ac-globulin, 0.5 cc. thromboplastin in a volume of 5 cc. with CaCl₂ added to a final concentration of 0.03 M. Dilutions of this mixture were rapidly prepared in 0.03 M Ca-buffer. Figure 1 shows these values plotted as clot-

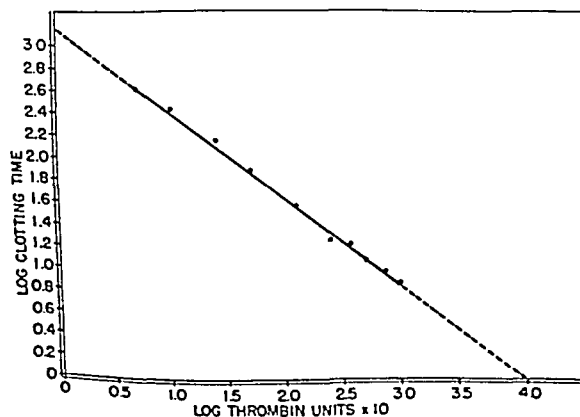


FIG. 2. LOGARITHM OF CLOTTING TIME PLOTTED AGAINST LOGARITHM OF UNITS OF THROMBIN $\times 10$

ting times against percentage thrombin. In Figure 2 are plotted the logarithms of the clotting times against the logarithms of the per cent thrombin $\times 10$. This gives a straight line which was interpolated to obtain values above 100. Values of less than 0.5 unit (C.T. = seven minutes) are grouped together as there is a little significance to minor variations in this range.

RESULTS

Controls:

Table I presents the control results for this series of experiments. Obviously, none of these materials are completely pure. The fibrinogen itself

TABLE I
Control results

Units of thrombin formed from thrombic mixture containing stated amounts of reagents in 5 cc.

T	Reagents*				Incubation period							
	Ca	Pro	AcG	Tpln	5 min.	15 min.	30 min.	60 min.	4 hr.	24 hr.	72 hr.	
1	.03	0.1	0.1	0.5	0.5	—	—	0.5	—	—	—	
2					0	0	0	0	0	0		
3					0.5	0.5	0.5	0.5	0.5	0	0	
4					0	0	0	0	0	0	0	
5	.03	0.1	0.1	0.5	0.5	—	0.5	0.5	0.5	0.5	0.5	
6	.03				0.5	—	0.5	0.5	0.5	0.5	0.5	
7	.03				0.5	—	0.5	0.5	0.5	0.5	0.5	
8	.03	0.1	0.1		0.6	0.6	0.6	0.6	0.8	0.6	0.5	
9	.03	0.1		0.5	0.5	1.0	1.5	2.4	8.0	8.0	13.0	
10	.03		0.1	0.5	1.5	3.5	4.5	4.5	5.0	5.0	4.5	
11	.03	0.1	0.1	0.5	30	67	89	100	100	84	70	

* Ca expressed as final molarity; other reagents in cc. of stock solution.

contains all the essential elements for coagulation except calcium and clots slowly (trace clot in one hour) on recalcification. This is enhanced slightly by addition of prothrombin, Ac-globulin or thromboplastin, but there is no evidence that these mixtures form thrombin themselves as there is no increase in thrombin activity during a 72-hour incubation period. This is likewise true of mixture 8, Ca + prothrombin + Ac-globulin. In contrast to this both mixtures 9 and 10 show thrombin formation suggesting (1) that either prothrombin or Ac-globulin is capable of producing thrombin, or (2) that these substances contain impurities. This problem is investigated in a later section.

Calcium effects:

Figure 3 presents the maximal thrombin yields of the same amounts of prothrombin, Ac-globulin and

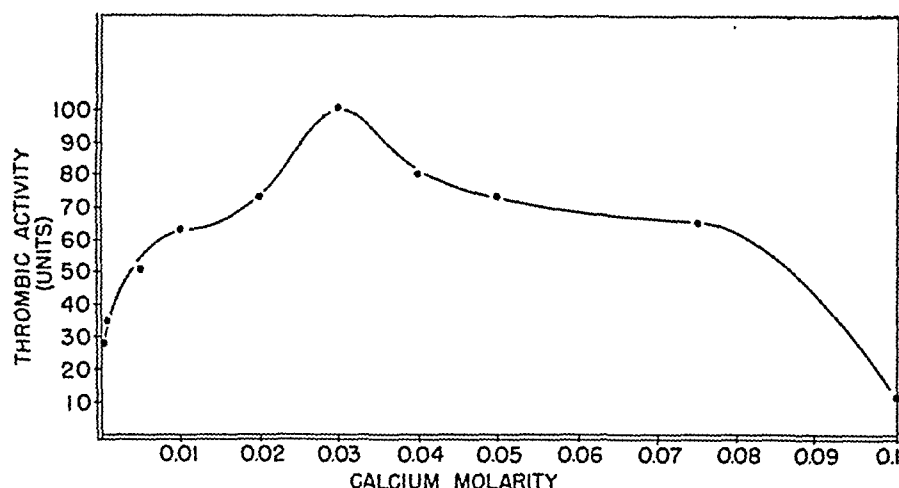


FIG. 3. MAXIMAL THROMBIN FORMATION AT VARIOUS CALCIUM CONCENTRATIONS (PRO, ACg AND TPLN CONSTANT)

TABLE II

Effect of calcium concentration

Units thrombin formed from thrombic mixtures containing 0.1 cc. 0.1% Pro, 0.1 cc. 0.1% AcG, 0.5 cc. Tpln in 5 cc. buffer at stated Ca concentrations.

Calcium molarity	Incubation period								
	5 min.	15 min.	30 min.	45 min.	60 min.	4 hr.	24 hr.	48 hr.	96 hr.
.0005	5.6	28	27	26	25	25	20	18	13
.001	18	35	35	35	30	28	28	27	16
.005	43	51	50	48	50	50	50	48	30
.01	51	63	61	58	58	58	52	51	34
.02	64	73	71	71	73	73	71	63	45
.03	30	67	89	100	100	100	84	80	51
.04	15	55	69	71	73	73	80	76	53
.05	7.5	29	53	60	61	62	69	73	54
.075	5	18	46	54	57	60	63	65	50
.1	0.6	0.9	1.1	1.5	2.5	4	10	11	11

thromboplastin in various Ca concentrations. The optimum Ca is a final concentration of 0.03 M.

Inspection of the raw data in Table II reveals that maximal thrombin formation is rapid in Ca concentration of 0.0005 to 0.02 M and thrombin yield increases with increasing Ca concentration. At 0.03 M the rate of thrombin production is somewhat slower, the maximum being reached at 45 minutes. Above this the rates are still slower and the final yield decreases as the Ca concentration increases.

An explanation for this inhibitory effect of high Ca concentration was sought. Table III shows that the major effect occurs in thrombin production and not in conversion of fibrinogen to fibrin.

Table IV shows that adjustment of the pH does not affect the Ca inhibitory effect.

TABLE III

Effects of Ca concentration on conversion of fibrinogen to fibrin

Calcium molarity	Clotting time (seconds)
0.0	8
0.001	9
0.01	9
0.03	8.5
0.1	8

0.25 cc. of purified thrombin (.001%) in various Ca concentrations was added to 0.5 cc. B.F.

Table V shows that increasing the ionic strength (as crudely measured by specific resistance) of 0.01 M Ca to that of 0.1 M Ca by addition of a neutral salt greatly slows the rate of thrombin formation but does not completely account for the Ca inhibitory effect.

We also wished to determine whether the calcium optimum varied with the prothrombin or Ac-globulin content. Thrombic mixtures containing prothrombin 0.01 cc. and prothrombin 0.5 cc., with Ac-globulin and thromboplastin constant at 0.1 and 0.5 cc. respectively, were prepared with various concentrations of Ca and the optimum in each case

TABLE IV

Effect of adjustment of pH on thrombin formation in high calcium concentrations

Calcium molarity	pH	Incubation period (minutes)				
		5	15	30	60	240
0.03	7.4	30	67	89	100	100
0.1	7.15	0.6	0.9	1.1	2.5	4.0
0.1*	7.45	1.0	1.0	1.1	1.5	3.0

* Adjusted with 1 M NaOH.

was found to be 0.03 M Ca. Thrombic mixtures containing 0.005, 0.01, 0.05, and 0.5 cc. of Ac-globulin with prothrombin and thromboplastin constant at 0.1 and 0.5 cc., respectively, were prepared. The Ac-globulin concentrations of 0.05 and 0.5 cc. reached maximal thrombic activity in Ca concentration of 0.03 M. In the lower Ac-globulin concentrations the Ca concentration had less effect, maximal yields being obtained in Ca ranges of 0.01 to 0.03 M with a more rapid thrombin formation in the lower Ca concentration.

Effects of Ac-globulin:

Figure 4 shows the effects of increasing concentrations of Ac-globulin. Both the rate of thrombin formation and the final thrombin yield were increased by increasing concentrations of Ac-globulin in thrombic mixtures containing constant prothrombin, thromboplastin and Ca content. No optimum Ac-globulin concentration was obtained above which additional thrombin was not formed.

TABLE V
Effect of adjustment of specific resistance
on thrombin formation

Calcium molarity	Specific resistance	Incubation period					
		5 min.	15 min.	30 min.	60 min.	4 hr.	24 hr.
0.1	40	0.6	0.9	1.1	2.5	4.0	10
0.01	128	51	63	61	58	58	52
0.01*	37.2	1.0	2.0	11	20	25	30

* Adjusted by addition of solid NaCl.

As the Ac-globulin alone forms about 5 units of thrombin from each 0.1 cc., at least this increase in thrombin production would be expected, as the Ac-globulin concentration is increased.

Thrombic mixture 9 (Table I), prothrombin + thromboplastin + Ca, showed the slow formation of 13 units of thrombin in 72 hours; longer incubation did not increase the yield. This suggested either that Ac-globulin was not necessary to thrombin formation or that the prothrombin or thromboplastin might contain Ac-globulin. Further purification was attempted by heating these substances. Table VI shows that both the thromboplastin and prothrombin lose some of their potentialities by heating in a boiling waterbath. The boiled thromboplastin and fresh prothrombin still form 6.0 units of thrombin. If the prothrombin is also boiled no appreciable thrombin is formed unless Ac-globulin is added. From this we must con-

TABLE VI

Effect of heat treatment on removal of AcG from Pro and Tpln

Reagents					Incubation period						
Pro I	Pro II	Tpln I	Tpln II	AcG	5 min.	15 min.	30 min.	60 min.	4 hr.	24 hr.	72 hr.
0.1	0.1 0.1	0.1		0.1	30	67	89	100	100	84	70
0.1				0.5	1.0	1.5	2.4	8.0	8.0	13.0	
0.1		0.1	0.1	22	63	69	84	80	75	70	
0.1		0.1	0.1	0.5	0.5	0.5	1.5	3.0	6.0	6.0	
0.1		0.1	0.1	1.0	2.5	8.0	8.0	14.0	22.0	16.0	
			0.1	0.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Pro I = untreated, Pro II = 100° C. for seven minutes, Tpln I = untreated, Tpln II = 100° C. for seven minutes. 0.03 M Ca throughout.

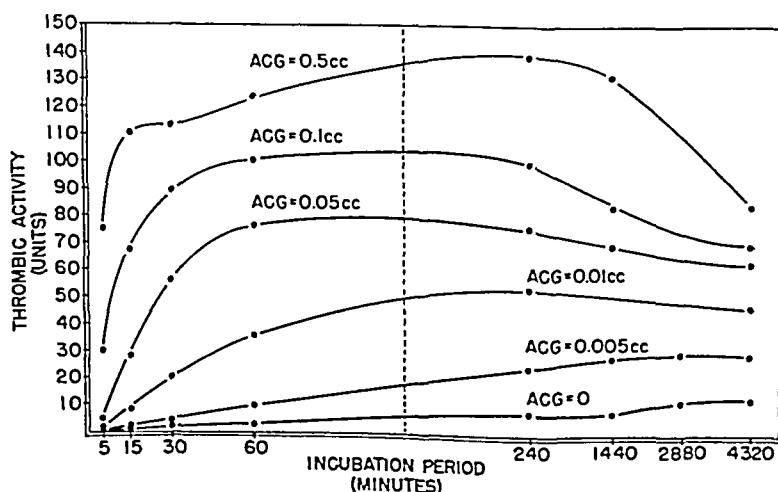


FIG. 4. EFFECTS OF VARYING QUANTITIES OF ACg (PRO, TPLN AND CA CONSTANT)

TABLE VII
Effect of BaSO₄ treatment on AcG

Reagents			Incubation period					
Pro	AcG	AcG(BaSO ₄)	5 min.	15 min.	30 min.	60 min.	4 hr.	48 hr.
0.1	0.1		30	67	89	100	100	80
0.1		0.1	0.8	2.0	5.0	10.5	21	15
	0.1		1.5	3.5	4.5	4.5	5.0	5.0
		0.1	0.5	0.5	0.5	0.5	0.5	0.5

All thrombic mixtures contain Ca .03 M and Tpln 0.5 cc.

clude that both prothrombin and thromboplastin contain traces of Ac-globulin which can only be removed by drastic treatment which also destroys some of their thrombin-forming ability.

Thrombic mixture 10 of the control series showed some thrombin formation from Ac-globulin without the addition of prothrombin. This suggested that Ac-globulin contained traces of prothrombin. In order to prove this we incubated the Ac-globulin with $\frac{1}{5}$ volume of 35% barium sulfate suspension for 10 minutes, removed the precipitate by centrifugation and tested it as shown in Table VII. No thrombin was formed in the prothrombin-free mixture but although much of the Ac-globulin activity was lost by barium sulfate treatment, enough remains to form some thrombin when prothrombin is added.

Effects of thromboplastin:

Figure 5 shows the effects of varying quantities of tissue thromboplastin on thrombin formation from fixed quantities of prothrombin, Ac-globulin and Ca. No thrombin is formed in the absence of thromboplastin. As the concentration of thromboplastin increases the rate of thrombin formation

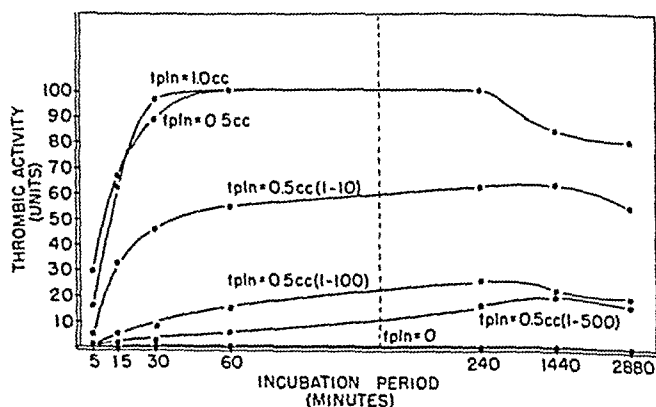


FIG. 5. EFFECTS OF VARYING QUANTITIES OF TPLN (PRO, AC G AND CA CONSTANT)

and yield of thrombin are increased to a maximal point above which no effect is noted on further increase of thromboplastin.

Effects of prothrombin:

Table VIII shows the effects of varying prothrombin concentration in mixtures containing standard amounts of Ac-globulin and thromboplastin. Unit values over 100 are calculated from Figure 2. When $\frac{1}{10}$ volume of prothrombin is used, roughly one-tenth the amount of thrombin is formed. When five-fold volume is used, roughly five times the amount of thrombin is formed.

TABLE VIII

Effect of varying Pro concentration

Units thrombin formed from thrombic mixtures containing 0.1 cc. 0.1% AcG, 0.5 cc. Tpln, and stated amounts of Pro in 5 cc. 0.03 M Ca-buffer

Pro cc.	Incubation period						
	5 min.	15 min.	30 min.	60 min.	4 hr.	24 hr.	72 hr.
0.01	8.7	11.5	11.5	11.5	11.5	12.4	11.8
0.10	30	67	89	100	100	100	70
0.50	38	403	553	600	634	634	496

DISCUSSION

An artificial system of purified reagents has been set up to study the formation of thrombin. Data obtained suggest that four substances—prothrombin, serum Ac-globulin, thromboplastin and calcium ions—are necessary for thrombin formation and that the yield of thrombin depends upon the proportions of these substances.

Inspection of our data reveals that thrombin, even that formed from these purified reagents, deteriorates rather rapidly. It is always possible that significant deterioration of thrombin has occurred before full activation in the slower thrombic mixtures.

If we again review our data in light of these considerations, we note that the rate of thrombin formation is rapid in calcium concentrations of 0.0005 to 0.02 M, that thrombin yield increases with increase of Ca, and that each of these mixtures loses about 10 units of activity in the ensuing 48 hours. It is difficult to conclude otherwise than that thrombin yield is directly dependent upon calcium concentration, although calcium optimum apparently varies little or not at all with prothrombin or Ac-

globulin content. The inhibitory effects of high calcium concentrations were in part explained as general effects of high salt concentration.

We were rather surprised to find our calcium optimum so much higher than that of other investigators (15, 16). This can in part be explained by the frequent use by many investigators of a one-stage prothrombin determination in which rate of thrombin formation is determined rather than the final yield of thrombin. Before determining the calcium optimum for this system, exactly similar preliminary experiments were performed at 0.0025 M Ca. The results obtained showed similar trends although the thrombin yields were somewhat lower. There is no evidence that the high Ca optimum is dependent upon excessive amounts of citrate or oxalate in these preparations. As far as we are aware there are only traces of these substances present.

Ac-globulin has been shown to increase both the rate of formation and yield of thrombin from a given amount of prothrombin, thromboplastin and Ca. In fact, our results indicate that Ac-globulin is necessary for the formation of any thrombin, but in order to demonstrate this we employed drastic methods (boiling) which destroyed a great deal of the prothrombin itself. We also tried heating at various temperatures below boiling but usually obtained prothrombin preparations in which some activation occurred without added Ac-globulin.

The Ac-globulin itself was noted to produce some thrombin when activated with thromboplastin and Ca. We attempted to remove the prothrombin from Ac-globulin by adsorption onto barium sulfate. In this way we were able to obtain an Ac-globulin which was completely free of prothrombin, but in the process we lost most of the Ac-globulin property.

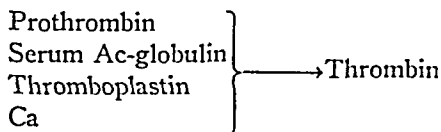
Thromboplastin was found to exert effects similar to Ac-globulin and Ca. Without tissue thromboplastin no thrombin was formed. As the concentration of thromboplastin increased the rate and yield of thrombin increased until a maximal amount was reached. Above this no effect was noted. These experiments have all employed thromboplastin derived from tissue. As this seems somewhat unphysiological, work is now in progress to study the effects of platelets and plasma thromboplastic substance.

Variations in prothrombin concentration produced marked changes in thrombin yield. When the prothrombin was reduced to $\frac{1}{10}$ the concentration of the standard mixture, and the Ac-globulin and thromboplastin maintained constant, thrombin was formed rapidly and about $\frac{1}{10}$ the final yield of thrombin was obtained. On the other hand, when the prothrombin was increased to five times the original concentration, thrombin was formed more slowly, complete activation occurring at four hours. The final calculated thrombin yield was somewhat greater than five times the standard yield.

From the experiments presented in this paper we are unable to formulate any absolute conception of the mechanism of blood coagulation. If it is assumed, as Seegers has done, that factor VI and serum Ac-globulin are identical, we are unable to confirm Owren's hypothesis that factor VI directly converts prothrombin to thrombin. If we then turn to Seegers' theory of thrombin formation we are forced to disagree in that we find serum Ac-globulin is necessary for prothrombin conversion, and our data indicate that thromboplastin, calcium, Ac-globulin, and prothrombin react together in such a fashion that the quantity of thrombin formed is dependent upon the quantity of each in the original mixture. We have as yet been unable to study the role of plasma Ac-globulin in this reaction. Previous reports (16) from this laboratory have suggested the formation of a thrombin "intermediary." How this hypothesis will be affected by the new factor, Ac-globulin, cannot be decided until more is known of the nature of this reaction.

CONCLUSIONS

From the data presented in this paper we conclude that four substances are necessary for thrombin formation:



None of these may be omitted, but we have not studied the substitution of other materials for these specific substances, *i.e.*, strontium for calcium, etc. The exact mechanism of interaction of these substances is not absolutely ascertained, but it appears

that the thrombin yield, as well as the formation rate, is dependent on the quantity of each.

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A STUDY OF ANTIFIBRINOLYSIN ACTIVITY IN THE PLASMAS OF VARIOUS ANIMAL SPECIES¹

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This report describes methods which have been developed in this laboratory for the determination of antifibrinolysin activity in plasma. Using these methods, studies have been made of the antifibrinolysin activity in the plasma of the alligator (*Alligator mississippiensis*), guinea pig (genus *Cavia*), cow (*Bos taurus*), rat (*Rattus norvegicus*, Sprague-Dawley strain), frog (*Rana pipiens*), man (white, male), cat (*Felis domestica*), dog (*Canis familiaris*, mongrel breeds), rabbit (genus *Lepus*, mixed strains), opossum (*Didelphis virginiana*), chicken (genus *Gallus*, Plymouth Rock), and pigeon (*Columbia livia*). With the exception of man, both sexes were represented but pregnant animals were not utilized in the sampling. Graphs are presented which indicate the relationships between plasma concentration (antifibrinolysin units) and the time required for the clots to dissolve. With the help of these graphs it is possible to measure the unit antifibrinolysin activity in a plasma sample from any of the species which have been studied.

The terminology used in this report will be the one recently suggested by Loomis, George and Ryder (1) and by Astrup and Permin (2). Considerable confusion has existed in the past regarding nomenclature and we believe that a uniform terminology will assist greatly in the development of this field.

Antifibrinolysin (Fibrinolysin Inhibitor [3], serum antitrypsin [4], antiplasmin [5], serum antiprotease [6]) is present in the plasma of all species which we have tested. It is not yet available in a pure state and therefore its chemical characteristics are unknown. This substance or substances in plasma inactivate fibrinolysin. It is not certain whether antifibrinolysin forms an inactive complex with fibrinolysin or whether the in-

activation is accomplished through alteration by antifibrinolysin of the fibrinolysin molecule.

Fibrinolysin (plasmin [7], serum tryptase [4], serum protease [6]) is produced in plasma by activation of a precursor(s), profibrinolysin (plasminogen [7], tryptogen [4], lytic factor [8]). The nature of profibrinolysin is unknown. The proenzyme may exist as an inactive fibrinolysin molecule (7) or it may comprise a complex which includes fibrinolysin and antifibrinolysin (5). Activation conceivably occurs through the separation of antifibrinolysin from the fibrinolysin molecule.

Three methods of activation of profibrinolysin have been described. (a) Streptokinase (termed fibrinolysin by Garner and Tillett [9]) is derived from certain strains of streptococci (10). When incubated with plasma or crude thrombin and fibrinogen, streptokinase promotes the production of proteolytic activity but streptokinase alone does not appear to have proteolytic activity (7). (b) Treatment of plasma or serum with chloroform has long been known to result in the production of an active enzyme (11). (c) Recently Astrup and Permin (2, 12) have demonstrated that tissue washings from the cells of various organs from several animal species will activate profibrinolysin. They have called this activator "cytofibrinokinase." Permin has also reported that the stroma of human erythrocytes contains an activator of profibrinolysin (12).

METHODS

1. *Preparation of special reagents.* The systems are buffered at pH 7.25 with imidazole. Ninety ml. of 0.1 N HCl are used to dissolve 1.72 g. of the salt. The pH is adjusted to 7.25 by means of HCl or NaOH and the solution made up to a 100 ml. volume with distilled water.

Thrombin Topical, Parke, Davis and Co., prepared by described methods (13-15), is employed

¹Aided by a grant from the U. S. Public Health Service.

in the clotting reactions. Two thousand Iowa units of dry thrombin are dissolved in a glycerol-water mixture containing equal parts by volume of water and glycerol. The glycerol is used for stabilization (16). This preparation is stable for several months when stored in a refrigerator at 5° C.

For use in assaying fibrinolysin or antifibrinolysin the thrombin solution is poured to a depth of about 1 cm. in a small test tube. Stirring rods, 3 to 4 mm. in diameter, are immersed in this solution. Upon adding fibrinogen to the test samples a clot is formed by removing the stirring rod from the thrombin solution and rotating it through the solution containing the fibrinogen. This insures that the fibrinogen is uniformly distributed throughout the enzyme solution. The stirring rod is then removed from the clotting mixture. It requires about two to three seconds to add thrombin in this manner; a firm clot forms within 15 to 20 seconds.

2. Preparation and standardization of fibrinogen. For use in quantitative measurements fibrinogen of high purity and reproducible clottability is required. The bovine fibrinogen employed in these assays was prepared by the freezing-thawing technic which has been described in detail (17). Since the preparation is stable under proper storage conditions, a large quantity is prepared and stored in a series of test tubes, each tube containing a sufficient quantity for performing the assays required during one day. The physically separated fibrinogen is dissolved to a concentration of 0.5 to 1.0 per cent in 2 per cent NaCl containing 5 per cent of the imidazole buffer. A sodium chloride concentration greater than 0.9 per cent and the slightly alkaline pH assist in stabilization. The fibrinogen concentration is measured by the determination of tyrosine (17, 18). After dividing this stock solution and placing it into a series of pyrex test tubes it is quick frozen in an alcohol dry ice mixture in preparation for storage at -20° C.

Prior to each series of fibrinolysin or antifibrinolysin assays, the fibrinogen solution is thawed at 40° C. without agitation and diluted to a fibrinogen concentration of 0.2 per cent in 0.9 per cent NaCl. This solution is maintained at 40° C. in a water bath except during pipetting procedures. If these precautions are followed, the fibrinogen

remains stable and the clottability is not measurably altered during at least a 24-hour period.

The fibrin concentration in the standardized clot has been fixed in these studies at 0.1 per cent because this concentration produces a firm clot which collapses rapidly at the end-point. Variations in fibrinogen concentration in the standardized solution result in appreciable errors in the antifibrinolysin assay. In Table I are given the dissolving times of clots, containing sufficient fibrinolysin to cause a 0.1 per cent fibrin clot to dissolve in 120 seconds, when the fibrinogen concentration is varied between approximately 0.5 and 1 per cent. The curve when plotted on arithmetical graph paper is a straight line (19).

TABLE I

Lysis time of clots formed from varying concentrations of fibrinogen. All other factors are standardized as described in the text

The 0.1 per cent fibrinogen and 120 second lysis time are used as the standard for fibrinolysin assays.

Per cent of concentration of fibrinogen in clot	Time required for clot to dissolve in seconds
0.046	86
0.092	111
0.139	133
0.185	166
0.231	189
0.278	216
0.370	268
0.463	310
0.648	413
0.926	575

3. Preparation and standardization of fibrinolysin solutions. The activity of the fibrinolysin supplied by E. C. Loomis and prepared by the method which has been described in detail (1) becomes stabilized in 15 to 20 minutes after it is dissolved in imidazole buffer and this activity remains relatively constant at room temperature for two to three hours thereafter. After three to four hours in solution at room temperature the activity gradually decreases. In view of these characteristics we permit about 50 minutes to elapse before attempting to standardize the fibrinolysin solutions and dissolve no more at one time than is required for a two to three hour series of assays.

For use in antifibrinolysin assays the unit activity of the fibrinolysin solution must be carefully standardized. We define one unit as the amount of fibrinolysin activity which will completely lyse 1 cc. of a 0.1 per cent fibrin clot at 28° C. in 120 seconds in an isotonic saline solution buffered with

TABLE II

Loss in activity of fibrinolysin solutions during 30 minute incubation at the indicated temperatures

Temperature degrees centigrade	Percentage loss in activity
45	90
40	85
35	75
30	17
28	0

imidazole. This unit requires three to five times as much fibrinolysin as the Loomis unit which is based upon the same dissolving time, but lyses a 0.3 per cent fibrin clot. Loomis, Ryder and George (1) carry out the lysis reaction at 45° C. instead of 28° C. Our reason for using the 28° C. temperature is based upon fibrinolysin inactivation studies. As indicated in Table II a large percentage of the fibrinolysin activity is lost in 30 minutes at 45° C. However, our tests have not included the shorter periods (two to five minutes) usually employed in the assay.

Figure 1 depicts the relationship between fibrinolysin activity and the breaking time of 0.1 per cent fibrin clots at 28° C. The relationship is a straight line function when plotted on log paper. It is similar to the relationship between thrombin concentration and clotting time (20). Thus the

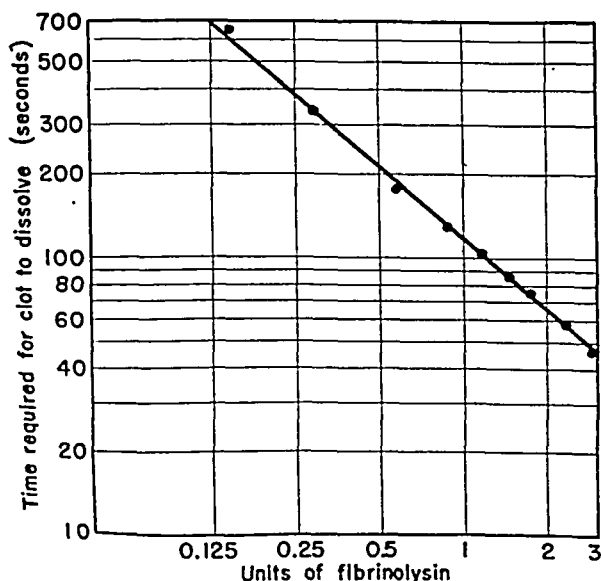


FIG. 1. RELATIONSHIP BETWEEN THE FIBRINOLYSIN CONCENTRATION AND THE DISSOLVING TIME OF A 0.1 PER CENT FIBRIN CLOT

All other variables are standardized as described in the text.

logarithm of clotting time is inversely proportional to the logarithm of the thrombin concentration and the logarithm of the lysis time is inversely proportional to the logarithm of the fibrinolysin concentration.

In preparation for a fibrinolysin or an antifibrinolysin assay a measured volume of the imidazole buffer is added to a weighed quantity of the dry fibrinolysin. Solution is facilitated by stirring. Thirty minutes after adding the imidazole buffer to the fibrinolysin the material is centrifuged at 3,000 r.p.m. for 20 minutes. This procedure removes undissolved particles. The supernatant solution is used for assay.

To determine the unit activity of a fibrinolysin solution (Figure 1), 0.2 ml. is pipetted into a test tube, 50 mm. \times 8 mm. I.D. As 0.2 ml. of 0.2 per cent fibrinogen is pipetted into the tube a stop watch is started. Thrombin is added by stirring rod as described above and the tube placed in a 28° C. water bath. Thirty seconds after adding the fibrinogen a capillary tube, 0.5 to 1.0 mm. I.D. with a beveled tip, is lowered gently down the side of the test tube. The capillary tube pushes the formed clot to one side and the beveled tip of the capillary tube rests on the bottom of the test tube. The stop watch is stopped at the end-point of the titration, when the liquid within the capillary tube passes the level of the surrounding liquid. The capillary tube end-point method was recently developed in this laboratory to eliminate errors which may occur from temperature changes and agitation when the method of tilting the tube is used to determine completion of lysis (19). The results which are given in this report, however, were obtained by means of the tilting method which gives essentially the same end-point but greater variation in duplicate and triplicate determinations.

The standardization of fibrinolysin for use in the antifibrinolysin assay is performed in the same manner except that the concentration of fibrinolysin in imidazole buffer is double that utilized in the fibrinolysin assay. One-tenth ml. of this solution is added to the reaction tube followed by 0.1 ml. of normal saline solution. Adjustment must be made in the fibrinolysin concentration to bring the activity, measured in this manner, to a lysis time of 120 ± 5 seconds. It is best to make the original fibrinolysin solution slightly more concentrated

and add normal saline in adjusting to the required activity.

4. *Collection and preparation of plasma.* In each species seven parts of whole blood were mixed with 1 part of 1.85 per cent potassium oxalate. Hematocrit determinations were made and the blood was immediately centrifuged to remove the cells. The plasma was defibrinated by adding Thrombin Topical, Parke, Davis and Co., in the dry form and the fibrin removed with a glass stirring rod. If the antifibrinolysin assay could not be performed immediately, the plasma was stored at -20°C . until the following day.

All animals used for obtaining blood samples were in a state of good nutrition and exhibited no obvious pathology. When anesthesia, ether or nembutal, was employed, as in the cat, dog and opossum, blood was taken immediately after the induction of anesthesia. Macfarlane and Biggs (21) have indicated that fibrinolytic activity occurs as frequently in the plasma of human patients during anxiety states as during anesthesia. We have not yet investigated the effect of anesthesia on the antifibrinolysin activity of plasma.

The antifibrinolysin assay curves are based upon the pooled blood from several individuals of each species. The number of individuals making up the pooled samples are as follows: alligator 3, guinea pig 6, cow 4, rat 10, frog 24, human 10, cat 4, dog 9, rabbit 12, opossum 3, chicken 6 and pigeon 6. In addition, determinations of antifibrinolysin in the plasma of individuals of several species (bovine, man, dog, chicken) indicated that the antifibrinolysin activity in the plasma of a normal individual closely approximates the value obtained from an analysis of the pooled plasma of that species.

5. *Assay of plasma antifibrinolysin.* Fibrinolysin is relatively stable in solution at pH 7.2 over a period of two to three hours if the temperature of the solution remains at 28°C . or below. However, if diluted plasma is added to the fibrinolysin solution, a portion of the fibrinolysin activity disappears rapidly. If the added plasma is sufficiently dilute, equilibrium is reached within five to 10 minutes and thereafter either no additional fibrinolysin activity disappears from the system or the decrease in activity is relatively slow. In Figure 2 is plotted time against lysis time for a series of diluted human plasmas. The time curves for chicken plasma are included in another report

(19). Although actual tests have not been made, all available evidence indicates that the antifibrinolysin in the plasmas of other animals acts in a similar manner.

The measurement of the amount of fibrinolysin remaining after 60 minutes in the presence of antifibrinolysin determines the concentration activity of the antifibrinolysin. More concentrated plasma (higher antifibrinolysin concentration) inactivates a large percentage of the fibrinolysin present while dilute plasma (lower antifibrinolysin concentration) inactivates less fibrinolysin. Therefore if fibrinolysin of standardized activity is allowed to react with antifibrinolysin until equilibrium occurs, a long dissolving time of a standard clot indicates a large amount of antifibrinolysin while a short dissolving time indicates a low antifibrinolysin activity. The assay of antifibrinolysin is based upon these considerations.

Five units of plasma antifibrinolysin are defined as the quantity of antifibrinolysin which within 60 minutes at 28°C . and at pH 7.2 will inactivate 50 per cent of the fibrinolysin in 1 ml. of a standard solution containing 1 unit of fibrinolysin per ml.

To 0.1 ml. of the double strength standardized fibrinolysin solution in a 50×8 mm. test tube is added 0.1 ml. of diluted plasma. The tube is then

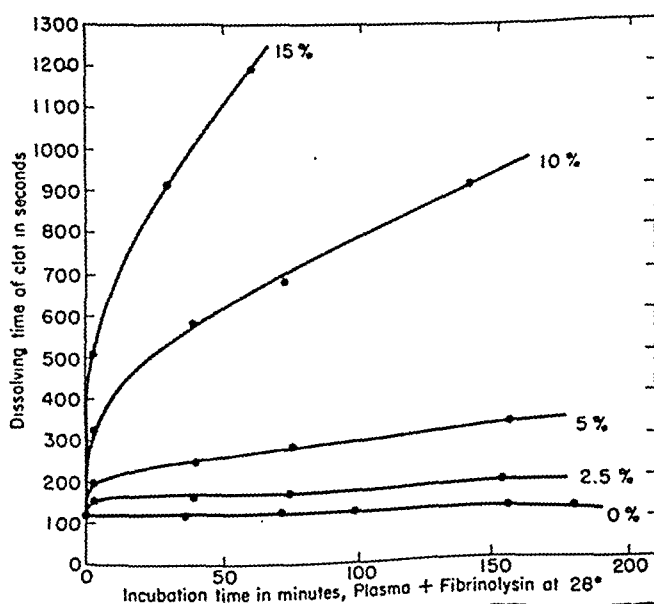


FIG. 2. THE DISSOLVING TIME OF STANDARDIZED CLOTS CONTAINING HUMAN PLASMA CONCENTRATIONS AS INDICATED ON EACH CURVE

The incubation time of fibrinolysin with plasma is measured on the abscissa.

placed in a water bath at 28° C. for 60 minutes. After 60 minutes' incubation the tube is removed from the water bath and 0.2 ml. of the standard 0.2 per cent fibrinogen solution added as the stop watch is started. Thrombin is added by stirring rod as above and the solution mixed. The tube is then returned to the water bath. The end-point is the complete dissolution of the clot (see No. 3 above). The elapsed

time between the addition of fibrinogen and the dissolution of the clot is recorded. All determinations are carried out in triplicate and are based on the average for the three measurements.

6. *Assay curves.* The curves in Figures 2 and 3 for the plasmas of the various animal species studied are based upon the plot of a series of plasma dilutions against the dissolving time of the standard clot. In each curve the coordinate

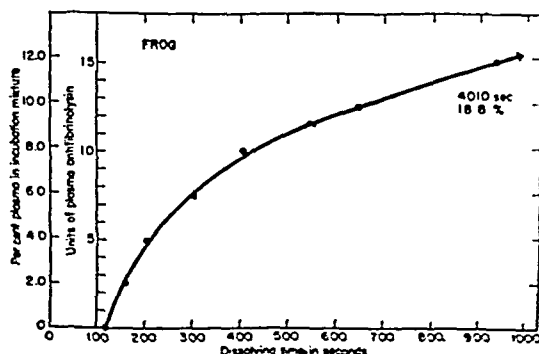
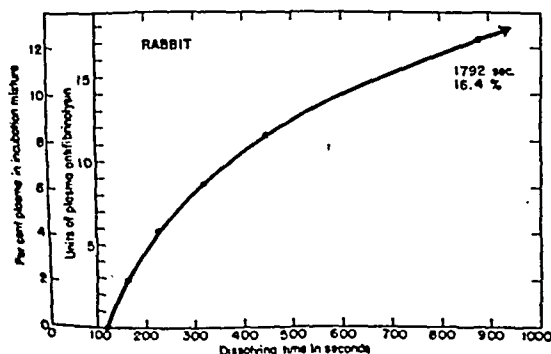
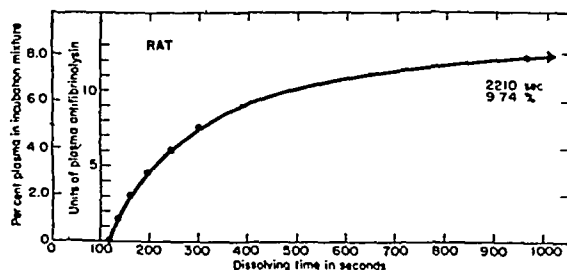
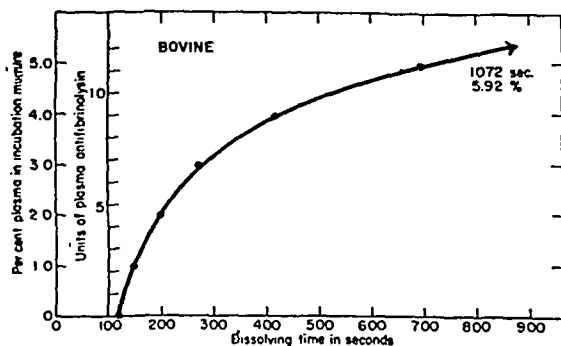
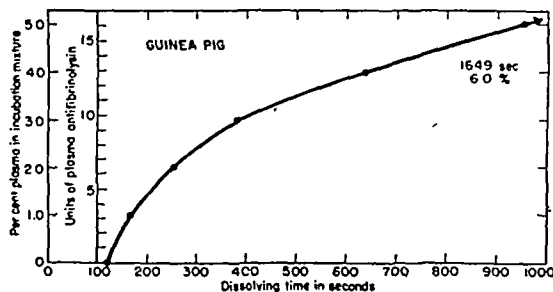
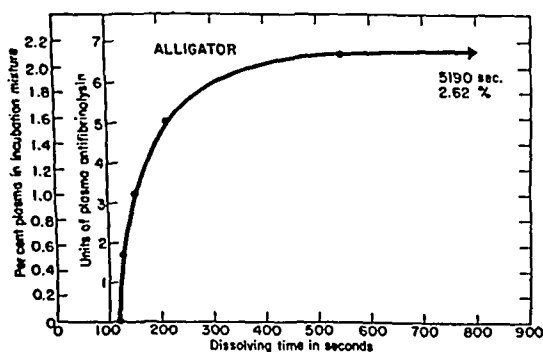


FIG. 3. THE DISSOLVING TIME OF STANDARDIZED CLOTS CONTAINING VARYING PERCENTAGES OF THE PLASMAS OF EACH SPECIES STUDIED
See text for details and interpretation.

for the 210 second dissolving time has been determined accurately. The 210 second dissolving time corresponds to one-half unit of fibrinolysin activity in Figure 1. The coordinate has therefore been extended from the 210 second dissolving time on the antifibrinolysin concentration curves to the ordinate and this value designated as 5 units of antifibrinolysin activity. The ordinate has been further divided into fractions and multiples of 5 units.

To assay the antifibrinolysin activity in the plasma of any of the species which we have studied, the plasma is obtained and prepared as described under No. 4 above. Dilutions of the plasma are made such that the dissolving time of the standard clot is less than 300 seconds. The plasma and the standardized fibrinolysin are incubated at 28° C. for 60 minutes. Fibrinogen and thrombin are added and the dissolving time of the clot determined at 28° C. By reference to the curve for

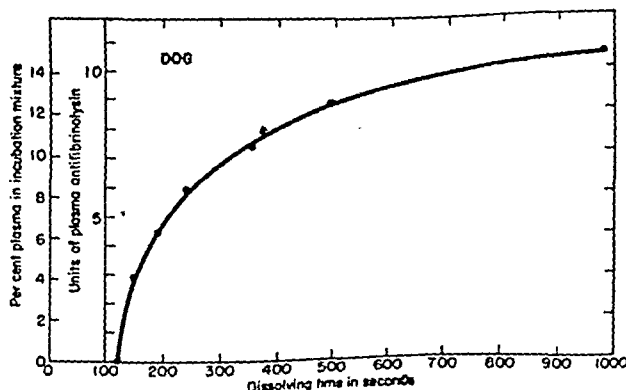
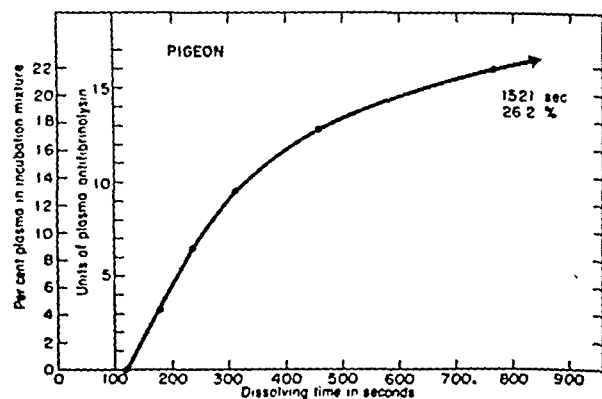
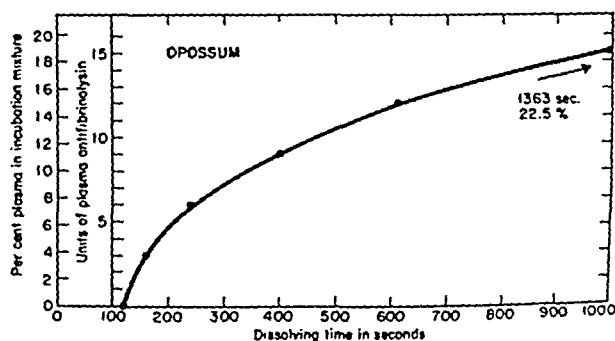
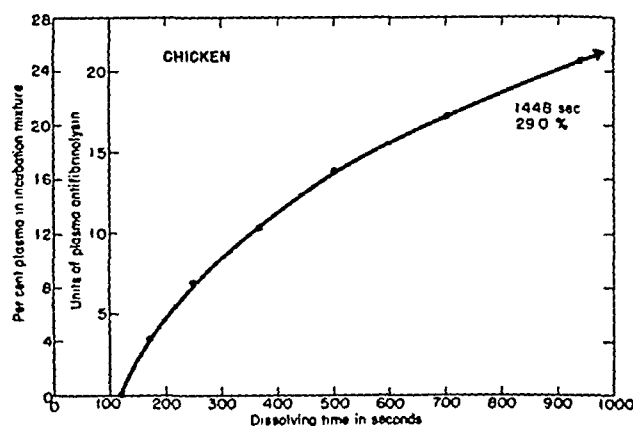
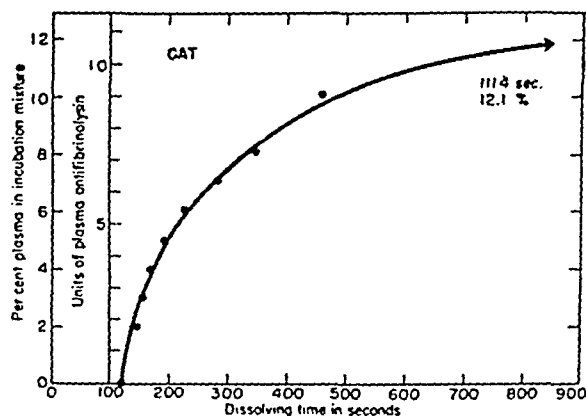
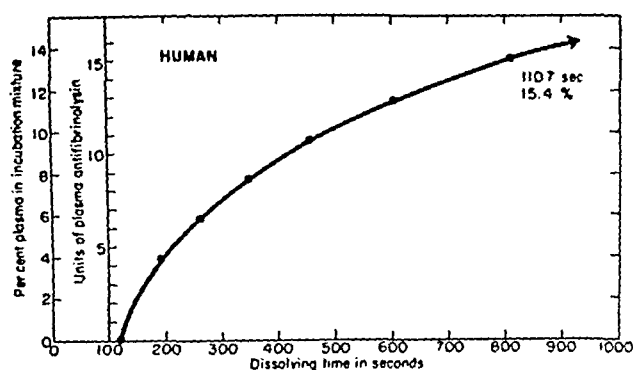


FIG. 4. THE DISSOLVING TIME OF STANDARDIZED CLOTS CONTAINING VARYING PERCENTAGES OF THE PLASMAS OF EACH SPECIES STUDIED

See text for details and interpretation.

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lysin is the substrate, alligator plasma is found to have the greatest antifibrinolysin unit activity per ml. of plasma and in decreasing order of activity the species are: guinea pig, bovine, rat, rabbit, frog, human, cat, chicken, opossum, pigeon, and dog. This order is based upon the plasma concentration at an antifibrinolysin activity level of 5 units. Slight variations in the species order would result from using higher or lower unit activities as the reference point. This discrepancy is the result of slight differences in the character of the curves from species to species.

DISCUSSION

The methods presented in this report make possible a comparative study of the antifibrinolysin activity in different animals and should prove to be useful tools in the determination of physiological and pathological factors which alter the concentration of this plasma component. In addition, experimental alteration of the plasma antifibrinolysin activity level may be followed through use of this assay method and the physiological significance of

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such changes in antifibrinolysin activity determined by observation of the effects upon a single tissue or upon the economy of the entire organism.

At the present time it is not practical to assay for the fibrinolytic activity of plasma. Methods which have been suggested (5, 22) require excessively long periods of time and indicate little more than the presence or absence of fibrinolytic activity in an *in vitro* system. Evidence obtained through the use of such methods indicates that free fibrinolytic activity may occur in plasma (5, 22-25). However, since an excess of antifibrinolysin in the plasma rapidly inactivates free fibrinolysin, an assay indicating no antifibrinolysin activity would be an indication that free fibrinolysin could exist. Our assays which include studies of the antifibrinolysin activity levels in several hundred animals of different species and under various experimental conditions as well as the plasma levels in normal and pathological human individuals have failed in all cases to indicate an absence of antifibrinolysin. It therefore appears that antifibrinolysin is commonly in excess. From available evidence it is not possible to ascertain whether plasmas which are fibrinolytically active result from an increase in fibrinolysin concentration or from a decrease in the antifibrinolysin activity.

The physiological significance of differences between species in plasma antifibrinolysin activity and in the character of the curves presented in Figures 3 and 4 cannot be determined from the available data. In the assay presented here, the factors existing in the plasma of different species are permitted partially to inactivate bovine fibrinolysin. The fibrinolysin molecule may differ in each species or it may be identical. In the same way antifibrinolysin may differ from species to species, or the same molecule may be present in the plasma of all species studied. It is possible that the relative antifibrinolysin activity in the plasmas of the different species would fall in a different order if the fibrinolysin were derived from the plasma of some species not belonging to the bovine genus. However, even though the fibrinolysin molecule may differ from species to species, the fact that the plasma of all species studied will inactivate bovine fibrinolysin makes possible comparative studies of antifibrinolysin activity in any one of the several species investigated.

SUMMARY

1. Methods are described which make possible the study of antifibrinolysin activity variations in the plasma of the alligator, guinea pig, cow, frog, rat, rabbit, man, cat, dog, opossum, pigeon, and chicken. The antifibrinolysin in the plasma of each species inactivates bovine fibrinolysin.

2. Fundamental relationships, important in the assay, are described. These include (1) the relationship between the fibrinogen concentration and the dissolving time of clots, (2) the inverse relationship between the logarithm of the fibrinolysin concentration and the logarithm of the dissolving time of the clots, (3) the equilibrium reaction between fibrinolysin and diluted plasma antifibrinolysin and (4) the character of the curves for the different species when the plasma concentration is plotted against the dissolving time of the clots.

3. Using bovine fibrinolysin as the enzyme the sequence of decreasing plasma antifibrinolysin activity by species is alligator, guinea pig, bovine, rat, rabbit, frog, human, cat, chicken, opossum, pigeon, and dog.

We wish to thank Eugene C. Loomis for generous supplies of fibrinolysin and Parke, Davis and Company for financial support of research in Physiology.

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A STUDY OF THE ANTIFIBRINOLYSIN ACTIVITY IN HUMAN PLASMAS DURING PATHOLOGICAL STATES¹

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Utilizing the methods described in the preceding report (1), a study of the antifibrinolysin activity in plasmas from patients of Receiving and Harper Hospitals in the City of Detroit has been initiated. The antifibrinolysin in human plasma which was assayed inactivates bovine fibrinolysin. The results indicate that there is a tendency for the antifibrinolysin activity to be increased in a number of different diseases. The conditions studied include: pneumonias, coronary thrombosis, pernicious anemia, secondary anemia, cirrhosis of the liver, intestinal obstructions and acute bacterial endocarditis. Other pathologies may also result in an increased plasma antifibrinolysin activity, although no increase was found in patients convalescing from fractures.

EXPERIMENTAL

Blood was obtained from the antecubital vein. Seven parts of whole blood were immediately mixed with one part of 1.85 potassium oxalate. Hematocrits were determined and the plasma separated by centrifugation. The plasma was immediately defibrinated by adding small increments of dry Thrombin Topical (Parke, Davis and Company). After each addition of thrombin a small portion of the plasma was tested for the presence of fibrinogen by the addition of a small amount of thrombin. If a clot formed or fibrin threads appeared another small amount of dry thrombin was added to the main plasma sample. When the plasma had been completely defibrinated the antifibrinolysin assay was performed immediately, or if this were not possible the plasma was stored at -20°C .

The antifibrinolysin assays were carried out as described in the preceding report (1). Plasma dilutions were made which brought the dissolving time of the standardized clots to between 200 and 300 seconds. From the dissolving time of the clot on the x axis (Figure 4 of preceding report [1]), the intercept with the curve was determined and the coordinate extended to the y axis from which could be estimated the unit activity of antifibrinolysin in the plasma. Correction was made in each

case for dilution and the addition of oxalate. Antifibrinolysin activity is expressed as units per milliliters of undiluted plasma.

RESULTS

Figure 1 is a graphical representation of the results. The plasmas of individuals exhibiting no obvious pathology were relatively uniform in antifibrinolysin activity. The mean for the normal group of six subjects was 79 units per ml. with the lowest value 59 units per ml. and the highest 98 units per ml. In contrast, marked variations in antifibrinolysin activity were found in plasmas from diseased individuals. In some cases the activity was found to be within the normal range, but extremely high values were obtained in other patients suffering from the same disease.

An individual in which intestinal obstruction was complicated by lymphogranuloma had the highest unit activity of antifibrinolysin in the plasma, 258 units per ml. In decreasing order of the mean antifibrinolysin activity unitage per milliliter of undiluted plasma the pathologies were

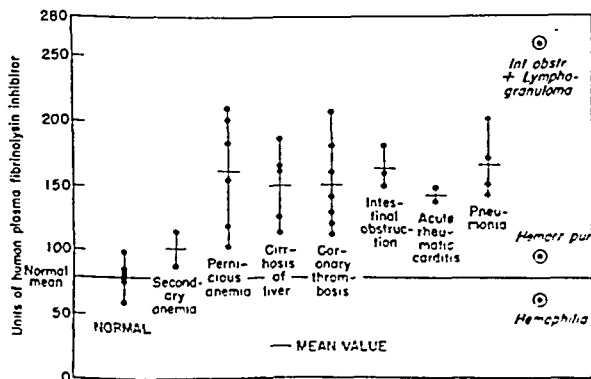


FIG. 1. GRAPHICAL REPRESENTATION OF PLASMA ANTIFIBRINOLYSIN LEVELS IN NORMAL AND DISEASED HUMAN INDIVIDUALS

The points indicate individual determinations; the short horizontal cross-lines the mean value for the group.

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pneumonias, intestinal obstruction, pernicious anemia, cirrhosis of the liver, coronary thrombosis, acute bacterial endocarditis and secondary anemia. However, in order to establish the relative levels a much larger series would be required. One case of hemorrhagic purpura and one of true hemophilia exhibited activity within the normal range.

DISCUSSION

The role of fibrinolysin and antifibrinolysin in the physiology and pathology of the animal organism has not been established. Evidence has been presented that active fibrinolysis occurs in shock (2) and that it takes place in the blood of cadavers in which death had occurred rapidly (3). In contrast patients suffering from acute streptococcal infections (4) and those exhibiting acute or chronic medical illnesses of a variety of types tend to have a higher than normal plasma antifibrinolysin activity. It has also been reported that the antistreptokinase activity of plasma increases during streptococcal infections (4, 5). Thus it appears that although a fibrinolytic tendency may occur in restricted circumstances, the more common reaction of the organism to injury involves an increase in the activity of those factors which tend to neutralize or prevent the formation of free fibrinolysin in the plasma.

Grob (6) has indicated that the ability to control leucoprotease (fibrinolysin?) and serum anti-protease (antifibrinolysin?) may be important and useful in the study and understanding of inflammations; protection of joint and other structures from proteolytic action; experimental arteriosclerosis and arterionecrosis; coagulation of blood; prolonging the action of insulin; release of thyroglobulin from the thyroid gland; bacterial growth and sulfonamide action; etc. It is possible that antifibrinolysin may have an additional function or functions in addition to the property of fibrinolysin inactivation.

The physiological consequence of an excess of free fibrinolysin in the plasma of the intact organism is difficult to determine. Fibrinolysin injected into a vein of a dog is rapidly inactivated by the excess antifibrinolysin in the plasma (7). From *in vitro* studies it is known that fibrinolysin inactivates prothrombin (8) and causes decomposi-

tion of fibrinogen and fibrin (9). Its effect upon other proteins, cell membranes, cell and vascular permeability and upon specific tissues is uncertain.

It is hoped that the assay method presented in this and the preceding report may offer a tool which will help in determining the significance of fibrinolytic and antifibrinolytic blood factors in the economy of the organism.

SUMMARY

The assay in human patients of the plasma anti-fibrinolysin which inactivates bovine fibrinolysin has revealed that this antifibrinolysin activity is increased in pernicious anemia, pneumonias, intestinal obstruction, acute bacterial endocarditis, and coronary thrombosis. One patient with true hemophilia and another suffering from hemorrhagic purpura showed no evidence of either an increase or a decrease in plasma antifibrinolysin activity.

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BLOOD VOLUME DETERMINATION IN THE HUMAN WITH RED CELLS CONTAINING RADIOACTIVE PHOSPHORUS (P^{32}) AND WITH PURE HUMAN ALBUMIN¹

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The work presented in this paper is an outgrowth of a previous study made in this clinic (1) and deals with the use of a relatively simple method by which red cells containing radioactive phosphorus (P^{32}) are used to measure blood volume in human subjects. Observations were also made on blood volume changes as measured by the effect of an intravenous injection of plasma and pure human albumin.

PREVIOUS OBSERVATIONS

Methods proposed for the determination of blood volume include both direct and indirect means. Complete historical reviews of these various methods have been reported by Keith, Rowntree and Geraghty (2) and by Erlanger (3). More recently Gregersen (4) and Gibson (5) summarized their results with the dye dilution method in normal and pathological conditions.

With the advent of radioactive tracer elements for use in biology and medicine, a new tool became available. Two radioactive isotopes (iron and phosphorus) have been employed to date. Radioactive iron (Fe^{59}) was first used by Hahn, Balfour, Ross, Bale and Whipple (6). Considerable data obtained by this method have been reported (6-14). The advantages of the radioactive iron lie in the fact that the activated red cells are stable for many weeks. The disadvantages are as follows: The need of a donor whose red cells have been synthesized with radioactive iron and whose blood has been carefully matched with the recipient's, the relatively large amount of blood needed, and the rather complicated procedure for determining radioactivity. Finally, in

the determination of red cell volume before and after hemorrhage or transfusion a second injection of donor red blood cells containing a different radioactive isotope of iron is necessary (8).

Radioactive phosphorus was used initially by Hahn and Hevesy (15). Two procedures have been described. The first, like the radioactive iron method, requires a donor animal whose red cells have been activated by the administration of P^{32} as Na_2HPO_4 . The second method, more recently developed by Nylin (16), uses the subject's own red blood cells which are activated and reinjected. The advantages of the second radioactive phosphorus method lie in the much greater simplicity with which P^{32} can be measured, the fact that no donor is needed, and that the volume of blood injected is small. The disadvantages lie in the short period (one hour) during which the red cells maintain constant radioactivity. Considerable data obtained by the two phosphorus methods have been reported (1, 15-26).

METHOD OF PROCEDURE

These methods will be described under two headings dealing with the use of radioactive phosphorus and of plasma albumin injections respectively.

I. The first method used, involving the radioactive red cells, was modified from that of Nylin (16). Into a clean, dry, 25-cc. pyrex tube were placed 1 to 2 cc. of a solution containing 50 microcuries of radioactive phosphorus.⁴ (Material with high activity was diluted to the

⁴ Radioactive phosphorus emits beta rays having a maximal energy of 1.8 and an average energy of 0.6 million electron volts. The maximum range of penetration of these rays through the body tissues is approximately 0.7 cm. The half-life time of P^{32} is 14.3 days (27). The 50 microcuries or less of P^{32} used in each determination of blood volume corresponds to less than 0.01 roentgen equivalents physical per day for 100 days. The accepted limit of tolerance for man is 0.1 roentgen per day (5, 28, 29). The exposure of the investigators to radiation was negligible and no extensive protective measures were required.

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same range of concentration with isotonic saline solution.) The mouth of the tube was plugged with cotton. All syringes and needles were washed thoroughly with distilled water. A 10-cc. pipette and a rubber stopper were similarly prepared. Since aseptic technic must be maintained throughout, all equipment was sterilized in an autoclave for 30 minutes at 15 lbs. of steam pressure.

From the subject, a sample of approximately 15 cc. of heparinized blood was withdrawn through the antecubital vein without stasis with a 19 gauge needle, and transferred to the test tube containing the radioactive solution. The tube was sealed with the rubber stopper and after mixing thoroughly by inversion was placed in an incubator maintained at 37° C., and agitated for two hours. A motor-driven stirrer with an eccentric shaft to which the samples were attached provided adequate mixing and prevented settling of the red cells.

At the end of the two-hour period of incubation and agitation, exactly 10 cc. of blood were removed from the tube with a pipette, transferred to a syringe, and injected into the subject. Blood was drawn back and forth into the syringe several times in order to insure the complete injection of all of the active material. Specimens were then removed at various intervals from the opposite arm after a five-minute period had elapsed to allow for adequate mixing. Samples of both the injected blood and that obtained subsequently were prepared for the determination of radioactivity by placing each specimen in hematocrit tubes of 8 mm. diameter and centrifuging them at 3000 r.p.m. for a period of 40 minutes. The plasma was then very carefully removed and replaced by distilled water to the original level. Resuspension of the cells in the water and subsequent hemolysis were accomplished by inverting or shaking the tube gently many times. (The originally incubated blood was obviously too active for direct counting; therefore after separation and hemolysis as described, it was diluted 1-500 and 1 cc. of this was used for counting.) One cc. of the hemolysate was placed on a pyrex watch glass and allowed to dry overnight in the absence of drafts. In order that the geometry of counting be maintained uniformly from sample to sample, the 1 cc. was confined to the area of a circle with a diameter of 25 mm. marked on the watch glasses with a china marking pencil. All the samples were counted at a uniform distance (2.5 cm.) below the counting window. All glassware was checked for contamination from previous samples. The samples were counted with a Geiger-Mueller counter for 15 to 30 minutes each. Background corrections were determined before and after each counting series. All counts were at least three times the background count. When repeated determinations of blood volume were made in the same individual one day or more later, an aliquot of the initial sample of blood, *i.e.*, the blood which was to be incubated with P^{32} was prepared and counted in like manner. The activity observed was subtracted from that present after incubation with P^{32} and from all subsequent samples following injection of the activated blood.

Inasmuch as the hemolysate was the same volume as that of the original whole blood, its activity is the same

as that of the red cells in 1 cc. of whole blood. This assumes that the activity of the phosphorus injected in the plasma fraction is independent of that in the red cells. That this assumption is actually correct within the limits of error of the experiment will be shown subsequently.

Calculation

The value for the total circulating blood is obtained from the following calculation. Activity is expressed as counts per cc. per minute after subtraction of background count.

$$W.B.V. = \frac{C_1}{C_2} \times V \quad \text{Formula 1}$$

Where:

W.B.V. is the whole blood volume

C_1 is the activity per cc. of hemolysate in the sample injected and

C_2 is the activity per cc. of hemolysate after injection

V is the volume of blood injected.

It will be noted that in the above calculation of whole blood volume the use of the measured hematocrit has been avoided. However, Formula 1 is based upon the assumption that the cell-plasma ratio is the same throughout the body. If this is not true, then the whole blood volume will not be accurately measured. For calculation of red cell and plasma volumes the hematocrit is used in the following calculations.

$$R.C.V. = \frac{C_1}{C_2} \times (V) \times \frac{H_2}{100} \quad \text{Formula 2}$$

Where:

R.C.V. is red cell volume

H_2 equals hematocrit of sample removed after injection.

After complete mixing of the injected activated red blood cells the ratio of activated to non-activated red cells is a constant throughout the vascular system. This ratio is independent of the cell-plasma ratio. Therefore the red cell volume as calculated according to Formula 2 is accurate since the use of the hematocrit value, in effect, converts the expression to one of the ratio of activated to non-activated red cells.

$$PV = WBV - RCV \quad \text{Formula 3}$$

Where PV = plasma volume.

Since, according to Formula 3, the plasma volume is calculated from the whole blood volume and the red cell volume, it is subject to the same possible inaccuracy as is Formula 1.

II. The second method of measuring blood volume by means of plasma and albumin injections was employed in patients with malnutrition, hypoproteinemia and edema. (Simultaneous whole blood, plasma and red cell volumes were also determined by the P^{32} method.) The procedure is based upon the changes in hematocrit and plasma albumin concentration following injection of a known amount of albumin, either as salt-poor human albumin (25 per cent) or as double strength plasma. Samples before and after the injection were collected and treated as already described. Fractionation was carried out by the method of Campbell and Hanna (30). Total and

fractional protein determinations were made by the colorimetric method of Weichselbaum (31).

Calculation of plasma volume involved the following formulae:

$$PV_0 = \frac{(\text{grams albumin injected}) \times 100}{A_1 \left(\frac{H_0}{100 - H_0} \right) \left(\frac{100 - H_1}{H_1} \right) - A_0} \quad \text{Formula 4}$$

Where:

PV_0 = plasma volume before injection in cc.

A_0 = concentration of plasma albumin before injection in grams per cent

A_1 = concentration of plasma albumin immediately after injection in grams per cent

H_0 = hematocrit before injection in per cent

H_1 = hematocrit immediately after injection in per cent.

Subsequent plasma volumes were calculated according to the following:

$$PV_1 = PV_0 \left(\frac{H_0}{100 - H_0} \right) \left(\frac{100 - H_1}{H_1} \right) \quad \text{Formula 5}$$

Where:

PV_1 = plasma volume after injection in cc.

PV_0 = plasma volume before injection in cc.

H_0 = hematocrit before injection in per cent

H_1 = hematocrit after injection in per cent.

FINDINGS

In Table I are listed the data obtained with radioactive red cells on nine normal individuals. This table also includes values for the blood volume and its fractions in normal men and women as obtained by several investigators using various methods (2, 4, 7, 18, 21, 32-36). It is readily apparent that our results are in close agreement with those obtained by Hevesy *et al.* (37), by Nylin and Hedlund (21), and by Govaerts and Lambrechts (18), who employed the same method in man, as well as the data presented by Gibson *et al.* (7) using the radioactive iron method. In the majority of instances the results obtained with P³² are lower than those obtained by the dye and carbon monoxide methods. This has also been noted with radioactive iron in man (Gibson *et al.* [7], Meneely *et al.* [13]) and in animals (10). Our results are somewhat higher

TABLE I

Eleven blood volume determinations using P³² in red cells in normal human subjects compared with findings of other authors*

			W.B.V.	R.C.V.	P.V.	W.B.V.	R.C.V.	P.V.
Mean			69.81	33.94	36.04	2631.7	1221	1410.5
Standard deviation			7.79	4.87	4.69	285.2	160	162.0
Standard error			2.36	1.47	1.42	86.2	48.4	48.9
Bibliographic reference of other authors	Method	Subjects						
(2)	Dye	42 m. & f.	cc./kilo. 85.0	cc./kilo.	cc./kilo. 50	cc./sq. m.	cc./sq. m.	cc./sq. m.
(32)	Dye	49 m. 41 f.	77.7 66.1	34.62 24.6	43.08 41.5	3019 2522	1399 1002	1620 1520
(4)	Dye		85±8.9		45.0±4.0			
(33)	CO	16 m. & f.	66.2			2467		
(34)	CO Dye	9 m. & f.	80.2±5.5 80.5±8.6	34.9 35.0	45.3 45.5			
(35)	CO Dye	20 m. & f.	71.0±5.0 93.0±8.0					
(36)	Dye Plasma	8				3350 3350		
(37)	P ³²	21 m. & f.		38.8				
(21)	P ³²	19 m. & f.		33.7				
(18)	P ³²		74					
(7)	Fe ⁵⁹	40 m.		29.7			1150	

* Key: W.B.V. = whole blood volume. R.C.V. = red cell volume. P.V. = plasma volume. m. = male. f. = female.

TABLE II
Blood volume determinations using P^{32} in red cells in patients*

Patient, age, sex	Diagnosis	W.B.V.	R.C.V.	P.V.	W.B.V.	R.C.V.	P.V.
		cc./kilo.	cc./kilo.	cc./kilo.	cc./sq. m.	cc./sq. m.	cc./sq. m.
B. S. m. 71	Carcinoma of pancreas, malnutrition, edema	50.5	22.9	27.6	1860	845	1015
G. S. m. 71	Cirrhosis of liver; no edema or ascites	85.2	32.6	52.6	2731	1044	1887
E. R. m. 75	Carcinoma of pancreas, malnutrition, edema	77.2	31.6	45.6	2820	1155	1665
B. P. m. 48	Cirrhosis of liver, ascites, anemia	76.9	23.3	53.6	2918	878	2039
G. H. f. 45	Non-tropical sprue, hypoproteinemia; generalized edema	57.8	24.2	33.6	2010	842	1168

* Key: W.B.V. = whole blood volume. R.C.V. = red cell volume. P.V. = plasma volume.

than those obtained by Meneely *et al.* (13) in their patients.⁵

In Table II are listed determinations made with the radioactive phosphorus technic in five patients with hypoproteinemia showing a variety of values from which few inferences can be drawn except to say that the procedure was fairly simple and was well tolerated.

Table III summarizes four representative cases in which the findings of plasma volume as obtained with the injection of salt-poor albumin or

TABLE III
Simultaneous plasma volume determinations
by P^{32} and albumin injection methods*

Case	Before injection of albumin		After injection of albumin	
	P.V. P^{32}	P.V. albumin	P.V. P^{32}	P.V. albumin
1	1942	2450	2480	2990
2	2000	2580	2580	3050
3	2997	3450	3672	4200
4	3246	3880	3902	4460

* Key: P.V. P^{32} = Plasma volume calculated from injection of red blood cells containing P^{32} , Formula 3.

P.V. alb. = Plasma volume calculated from changes in albumin concentration and hematocrit following injection of a known amount of albumin, Formulas 4 and 5.

Note that while the initial plasma volume is 500 cc. lower, as measured with radioactive phosphorus, the increase after the injection of albumin is about the same with each method.

⁵ Their findings are not included in Table I because it was impossible to calculate their data in the same manner since the body weight and surface areas were not reported.

plasma (Formula 4) were compared with those as determined by the P^{32} method. The former gave values which were usually 500 to 600 cc. higher. However, the increase in plasma volume following injection of albumin or plasma as determined by P^{32} was in close agreement with the change calculated from Formula 5. No significant change in red cell volume was observed by either method.

TABLE IV
Distribution of P^{32} in red blood cells of plasma, liver and skeletal muscle after injection of activated whole blood*

Patient	Days after injection of P^{32}	R.B.C.	Plasma	Liver	Muscle
		cts./cc.	cts./cc.	cts./gm.	cts./gm.
B. S.	2	640	30	589	30
E. R.	1	1160	25	820	208
Y. S.	5	82	10.5	234	162

* Key: cts. = counts.
R.B.C. = red blood cells.

Aside from the data on blood volume, other observations were made on three of the patients in whom we injected radioactive blood, who were operated upon one, three, and five days afterwards. In them it was possible to obtain liver and rectus muscle biopsies, as well as blood samples. The findings are recorded in Table IV, which shows that the liver and muscle tissue contain considerably more P^{32} than the plasma, but less than the red cells in two of the three cases. After such a time interval the P^{32} would probably

be contained in the phosphatide fraction of the red cells, plasma and liver rather than the acid soluble fractions (19).

DISCUSSION

The behavior of the phosphorus under the conditions of our study is obviously important. Much light has been shed on the subject by many investigations. The uptake of inorganic phosphorus by the red blood cells has been studied *in vitro* and *in vivo* in man and in animals by the use of both quantitative and tracer methods. In 1940 Hahn and Hevesy (15) demonstrated that P^{32} injected subcutaneously into rabbits entered the acid soluble fraction (hexose monophosphates and adenine triphosphate), and the phosphatides of the red blood cells. In short term experiments, the uptake of phosphorus into the carbohydrate cycle is much more significant than that into the phosphatides (19). Factors concerned with the uptake of phosphorus into the carbohydrate cycle of the red blood cells are:

1. *Time*: Lawazek (38) demonstrated that it required two to three hours for the glucose of red blood cells to be broken down to lactic acid.

2. *pH*: Alkalinization favors the synthesis, and acidification the breakdown, of phosphate esters [Halpern (39), Rapoport and Guest (40), and more recently Tulin, Danowski, Hald and Peters (41)].

3. *Temperature*: Halpern (39), Eisenman, Ott, Smith and Winkler (42), and Hahn and Hevesy (19) have shown that there is little or no uptake of phosphorus by the red blood cells at 0°C ., whereas the uptake at 37° is considerable.

We have found that, following incubation of whole blood with P^{32} for two hours at 37° , the activity of P^{32} in the red blood cells is greater than that of the plasma. This difference in the distribution of P^{32} was the same in the control subjects and patients with various diseases studied (see Table V).

Hahn and Hevesy (19) offer further evidence that the uptake of phosphorus by the red blood cells is actually due to metabolic activity by demonstrating that the addition of KCN to the system reduces markedly the formation of organic phosphorus compounds; that the uptake of phosphorus is independent of concentration of phos-

TABLE V
*Distribution of P^{32} between red blood cells and plasma after incubation for two hours at 37°C .**

Experiment†	Subject	R.B.C.	Plasma	Ratio:
				cts./cc. R.B.C. cts./cc. plasma
		cts./cc. $\times 10^6$	cts./cc. $\times 10^5$	
1	D. S.	4.88	3.08	1.58
2	D. S.	6.42	2.94	2.18
3	F. K.	4.85	5.55	0.87
4	F. K.	17.50	20.55	0.85
5	M. R.	2.91	1.76	1.65
6	C. R.	3.03	1.30	2.33
7	C. R.	15.72	12.25	1.29
8	A. H.	3.10	2.58	1.20
9	R. E.	4.27	0.95	4.50
10	R. K.	2.80	0.73	3.70
11	J. M.	4.99	2.40	2.08
	Mean			2.02
12	B. S.	7.68	4.91	1.57
13	B. S.	7.12	4.80	1.48
14	G. S.	3.46	2.26	1.53
15	J. H.	2.04	1.88	1.08
16	E. R.	6.71	4.33	1.55
17	E. R.	8.15	4.25	1.92
18	B. P.	4.18	2.27	1.84
19	B. P.	9.80	6.24	1.57
	Mean			1.69

* Key: same as Table IV.

† Experiments 1 to 11 inclusive were normal controls, 12 to 19 patients with hypoproteinemia.

phorus in the plasma; and that hemolysate at 37° takes up P^{32} , though at a slower rate than intact red blood cells.

In general, then, it may be said that the phosphorus enters the red blood cells in a large part by a process of organic synthesis (*i.e.*, metabolic activity) rather than simple diffusion according to concentration gradients and that the metabolic process primarily concerned is glycolysis during which organic phosphorus esters are synthesized. That the phosphorus taken up by the red blood cells during incubation is held within the red blood cells at a constant level for sufficient time to allow for mixing of the injected blood and subsequent sampling is demonstrated below. The reason the P^{32} that has entered the red blood cells does not begin to leave the red blood cells immediately after injection is not known.

The method used herein of measuring blood volume by means of P^{32} is subject to the following four sources of error, each of which is discussed in detail. (1) Error of hematocrit, *i.e.*, plasma adherence to packed red blood cells. (2)

Loss of P^{32} from the red blood cells within the time of determination of the blood volume. (3) Intrusion of P^{32} from the plasma into the red blood cells after injection (since activated whole blood is injected). (4) Hemolysis of the injected blood cells.

1. The error of the hematocrit due to adherence of plasma to the packed red blood cells has been studied and is found to be about 2 to 3 per cent (16, 19, 20, 43). Now if the plasma adherent to the red blood cells in the aliquot of the injected blood has the same activity as the red blood cells, there is no error in determination of C_1 in Formula 1. After injection and mixing time have passed, however, there will be an error

in the determination of blood volume in the positive direction, *i.e.*, the counts per cc. of sample after mixing (C_2 in Formula 1) will be lower than if no plasma were adherent because the activity of the plasma has fallen to about 20 per cent of the activity at zero time. As time passes this error will increase.

2. The error due to loss of P^{32} from the cells to the plasma is in the order of 1 to 2 per cent (1, 17, 19, 20, 37). This will also render the error in determination of blood volume positive because the counts per cc. (C_2) of blood after mixing will be lower than at zero time. It has been shown *in vitro* by Hahn and Hevesy *et al.* (19, 20, 37) and by Brown, Hempelman and

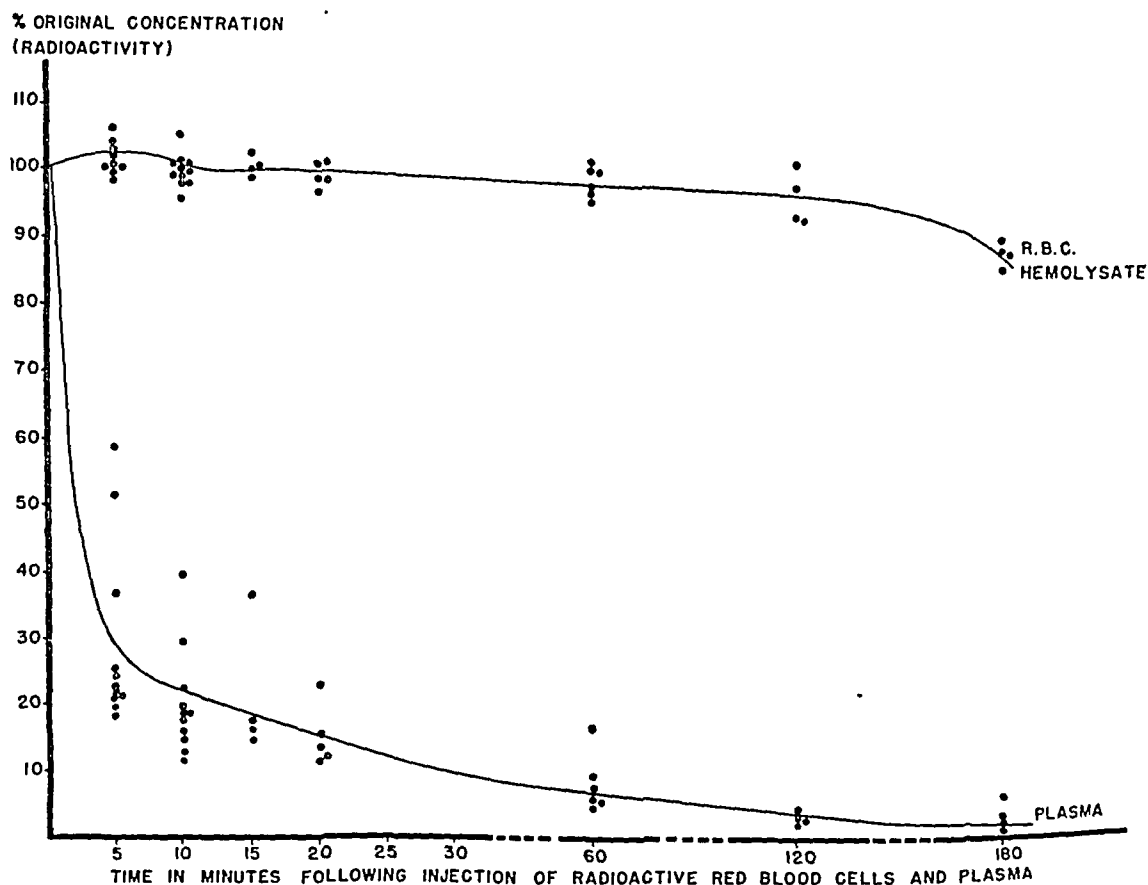


FIG. 1. RADIOACTIVITY OF RED CELLS AND PLASMA AFTER INJECTION

Each point represents a single determination of the radioactivity present in the red cells and in the plasma of 1 cc. of whole blood at various time intervals following the injection of the activated sample. The activity is expressed as a percentage of the theoretical activity at zero time which is set at 100 per cent. In the case of the red cells this zero value was obtained by extrapolation from the values at five, ten and 15 minutes. In the case of plasma this zero value was calculated from the plasma volume as subsequently measured together with the known amount of activity present in the injected plasma.

Note the consistency with which the activity in the red cells is maintained for a period of one hour with but little loss at two hours. By contrast note the almost immediate disappearance of the activity in the plasma which within 20 minutes had fallen to a maximum of 20 per cent.

Elman (1), that if red blood cells activated either by shaking in a thermostat at 37° or by repeated injection into a donor animal are mixed immediately with inactive NaCl, plasma or whole blood, there is little loss of activity over the period of one hour. Nylin (16, 21) has shown that in "normal" men there is no loss of activity of the red blood cells from 60 seconds to one hour after injection. We have found that there is no significant loss of activity from five minutes to one hour, and only a small loss in the second hour (Figure 1).

3. The error due to intrusion of P^{32} from the plasma into the red cells after injection would render the error in the determination of blood volume negative because the counts per cc. of red blood cells after mixing (*i.e.*, C_2) would be higher than at zero time. This error is thought to be in the magnitude of 3 to 4 per cent by Hahn and Hevesy *et al.* (19, 20, 37). Now if the activity of the red blood cells and plasma injected is equal and the ratio of P^{32} : P^{31} atoms in the plasma is ten times the ratio in the red blood cells (the acid soluble phosphorus concentration in red blood cells being ten times as great as in the plasma) the probability that P^{32} will pass into the cells rather than out is present until the activity of the plasma falls to 10 per cent of that at zero time. We have found that this point is reached in 20 minutes when the activity of the plasma has fallen to about 10 per cent of its activity at zero time. At this time the probability that P^{32} will flow into the red blood cells is equal to the probability that it will flow from the red blood cells to the plasma (the ratio of P^{32} : P^{31} in the plasma and red blood cells is now equal). From this point on, then, the error in determination of blood volume is that discussed above under Section 2, *i.e.*, the error due to loss of P^{32} from the red blood cells after injection. Nylin (16), however, found no significant change in activity of the red blood cells of normal men up to 60 minutes after the injection of either activated whole blood or activated red blood cells which had been washed and resuspended in inactive plasma.

4. The error due to hemolysis of the injected red blood cells has not been studied; however, the same principles apply as discussed under Section 2, *i.e.*, the loss of P^{32} from the red blood cells

into the plasma. That errors 1, 2 and 3 almost cancel out for a single determination is shown by the following table taken from Hevesy (37).

Estimate of different errors of experiments in determination of the erythron (Man)

Time (minutes)	Per cent error due to adherence of plasma to corpuscles	Per cent error due to intrusion of P^{32} from plasma into RBC	Per cent error due to loss of P^{32} by corpuscles
10	+5	-5	+1.5

Nylin (16, 21) has found no significant loss of activity within one hour and we have corroborated these results, indicating that this method may be accurately applied in man for both routine determinations and accurate estimations of changes in blood volume occurring within one hour.

It should be emphasized that the primary method of calculation herein described, Formula 1, gives a value for whole blood volume without using the hematocrit. It is, however, based upon the assumption that the cell-plasma ratio is the same throughout the vascular system. Nevertheless, when the hematocrit is used for measuring red cell volume (Formula 2) the results obtained are in close agreement with those of other investigators (7, 21, 37). Moreover, the following data (Table VI) concerning the measurement of red cell volume before and after phlebotomy demonstrate the accuracy of the red cell volume calculation. These data were obtained in four normal subjects bled 500 cc. The whole blood volume was measured, according to our modification of the radioactive phosphorus technic, as described above, before, immediately after, and one-half hour after the bleeding. The red cell volume and plasma volume were calculated from the hematocrit at these times. We found that the red cell volume determined after hemorrhage was within 5 per

TABLE VI
Red cell volumes before and after hemorrhage

Subject	Initial red cell volume	Red cells removed	Expected red cell volume	Red cell volume determined	Per cent error
	cc.	cc.	cc.	cc.	
D. S.	3010	252	2758	2900	4.98
R. K.	2220	227	1993	2055	3.1
F. K.	2535	258	2277	2215	2.73
C. R.	2040	241	1799	1838	2.17

cent of the expected red cell volume, in each case, and within 3 per cent in three of the four cases. The differences between the data obtained immediately after phlebotomy and one-half hour later were less than 2 per cent in all instances. Similar findings were reported by Gibson *et al.* (8), who employed radioactive iron in dogs and in one patient with secondary polycythemia, and found the red cell volume before hemorrhage equal to red cell volume after hemorrhage plus the red cell volume removed (plus or minus 3 per cent). They also observed that the red cell volume before transfusion was equal to the red cell volume after transfusion minus red cell volume infused (plus or minus 2 per cent).

Much has been written about the inaccuracy of the hematocrit (other than the error incurred because of the adherence of plasma to the packed cells). The pivotal question in the literature is whether or not the hematocrit as drawn from one portion of the body represents the true cell plasma ratio of the entire circulating blood. Smith *et al.* (35), Hahn *et al.* (10), Stead and Ebert (43), Gibson (5), Hevesy *et al.* (37), and Gibson *et al.* (9) all state that the hematocrit is not a true indication of the cell plasma ratio of the circulating blood. The opposite is expressed by Hopper *et al.* (34, 44) who produced evidence that the hematocrit is essentially accurate.

The experimental data presented by most workers to disprove the validity of using the hematocrit consist of determinations of the red cell volume from the dye plasma volume and the hematocrit before and after hemorrhage. Invariably they found that the red cell volume before hemorrhage is greater than that after hemorrhage plus the volume of the cells removed. They conclude that the hematocrit is therefore responsible for this discrepancy on the assumption that the dye method gives the correct figure for the plasma volume. This assumption may not be justified.

Methods employing the injection of plasma for the measurement of blood volume have been used for many decades. One of the more recent studies is that of Hopper (34, 44) who reported that the calculation of plasma volume in dogs from formulae similar to those used herein gave results which not only failed to check the figures for the dye or carbon monoxide methods, but often varied in the opposite direction. On the other hand, plasma

transfusions were used in a similar way in human subjects by Phillips *et al.* (36), who calculated plasma volume from the changes in specific gravity of the blood as well as from the changes in hematocrit or in the hemoglobin concentration and obtained similar results by both calculations; their results were in close agreement with simultaneous dye plasma volume methods.

In spite of these possible sources of error, it is believed that the method herein described is as accurate as any other now available and it is far more convenient for routine use than the other available refined methods. Therefore, it may prove of considerable clinical value in the study of patients suffering significant alteration in the blood volume or its constituents. It has proved useful in our experience in following changes in the total circulating red cells and plasma proteins in contrast to simple measurements of their respective concentrations. This has provided a three dimensional picture of the blood changes in various blood deficiencies, particularly following various types of intravenous therapy. A summary of its use in evaluating the respective effects of plasma and pure albumin injections in patients with chronic hypoalbuminemia is described in another report from this laboratory.

SUMMARY

1. Blood volume was determined in normal human subjects and in patients with chronic hypoalbuminemia, using red blood cells labeled with radioactive phosphorus (P^{32}). The method consisted of incubating a small sample of the subject's blood with an isotonic solution containing approximately 50 microcuries of radioactive phosphorus and reinjecting a portion thereof. With this technic a direct value for the whole blood volume is obtained. The plasma and red cell volumes are then calculated by means of the hematocrit.

2. Blood volume was also measured in five patients using values obtained from changes in the hematocrit and plasma albumin concentration following injection of a known amount of pure salt-poor albumin. When compared with the simultaneous determinations made with radioactive red cells, the former method gave higher results for initial plasma volume, but subsequent changes checked well.

3. Both methods proved relatively simple and would seem to be well adapted to clinical investigation.

4. The findings were in close agreement with those obtained by other investigators using similar and other methods.

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THE SPECIFICITY OF IMMUNE HUMAN SERUM ANTIHYALURONIDASE¹

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In the light of present knowledge there appear to be two kinds of hyaluronidase inhibition by blood serum. One inhibitor of hyaluronidase has been demonstrated in the serum or plasma of various normal, nonimmunized animals and man (1-6). Haas has reported that this inhibitor is not specific for any particular hyaluronidase, and that it is not an antibody but is an enzyme (3). However, recently Hadidian and Pirie have favored the impression that this inhibitor is analogous to an antitoxin or an antibody (5), and Dorfman, Ott, and Whitney have presented evidence (6) which contests some details of the report of Haas (3). The findings of Dorfman, Ott, and Whitney lend no support to the thesis that this inhibitor is an enzyme, and leave open the question as to whether or not this inhibitor in normal serum specifically antagonizes particular hyaluronidases (6). The other inhibitor of hyaluronidase has been demonstrated in the serum of rabbits which had been immunized with a purified hyaluronidase preparation (7-11). This inhibitor in immune serum specifically antagonizes the particular hyaluronidase which was used as the antigen; it is developed by an immune response to an antigenic stimulus and may be called an antihyaluronidase.

The inhibitor of hyaluronidase in normal, non-immune serum is thermolabile (3, 5, 6), whereas the antihyaluronidase of immune serum is thermostable (12).

A previous study demonstrated rises in titer of serum antihyaluronidase antagonistic to pneumococcus hyaluronidase in human patients following pneumococcus bacteremia (12-14), and some inhibition of pneumococcus hyaluronidase by the serum of every one of 50 normal human beings tested (12, 13). Only relatively low dilutions of normal sera inhibited the pneumococcus hyalu-

ronidase, whereas relatively high dilutions of patients' sera at the peak of immune response inhibited the pneumococcus hyaluronidase (12). The present study demonstrates that this immune response of human antihyaluronidase serum is specifically antagonistic to the hyaluronidase of the particular species of organism from which the immunizing material was derived (15).²

METHODS

Serial sera of patients who suffered bacteremia due to various kinds of bacteria were titrated against similar strengths of various hyaluronidases. Serial dilutions of each serum to be tested were incubated with a constant amount of one hyaluronidase, and these incubates were tested for residual hyaluronidase activity by Byers' modification (16) of McClean's mucoprotein clot-prevention (M.C.P.) test (11). The highest dilution of the serum which inactivated the constant amount of the hyaluronidase was taken as the antihyaluronidase titer of that serum, referable to the particular hyaluronidase used in the test.

The hyaluronidases used in these tests were unrefined filtrates of 72-hour cultures of *Pneumococci* Type 1, Type 2, and Type 7; *Hemolytic Staphylococcus aureus*; *beta* *Hemolytic Streptococcus*, and *Clostridium perfringens* (Cl. Welchii);³ and purified bovine testicular hyaluronidase.⁴ The hyaluronidases of the gram positive cocci were prepared by culture in beef heart infusion broth (Difco) which contained 0.75% potassium hyaluronate and no glucose. Before inoculation this medium was sterilized in the autoclave at ten pounds pressure for 15 minutes. The Cl. Welchii hyaluronidase and the potassium hyaluronate were prepared by the method of Byers, Tytell and Logan (17).

² This was reported briefly in *Federation Proc.*, 1948, 7, 282.

³ We are indebted to Dr. A. A. Tytell who prepared and supplied the Cl. Welchii hyaluronidase used in these tests.

⁴ The purified bovine testicular hyaluronidase was supplied by the courtesy of Dr. R. L. Mayer of Ciba Pharmaceutical Products, Inc. This hyaluronidase was purified by ammonium sulfate fractionation with subsequent dialysis and lyophilization according to the method of L. Hahn, *Biochem. Z.*, 1943, 315, 83, and contained 105 Viscosity Reducing Units per mgm.

¹ Supported by a gift in the memory of the late Ben L. Heidingsfeld, and by a grant from the Smith, Kline, and French Laboratories, Philadelphia, Pa.

The constant amount of each hyaluronidase used throughout these tests was 0.25 cc. of the unrefined hyaluronidase which had been diluted so that either a 1:8 or a 1:16 dilution⁵ prevented the mucoprotein clot, regardless of the original potency of the hyaluronidase preparation used. The constant amount of the bacterial hyaluronidases was essentially equivalent to the constant amount of the purified bovine testicular hyaluronidase, which contained 0.0013 mgm. of the purified hyaluronidase. This dilution of each hyaluronidase was freshly prepared each day that tests were done. All dilutions of hyaluronidase and of serum to be tested were made in 1% proteose peptone in physiologic saline.

Serum antihyaluronidase was determined in exactly the same way as described in the previous study (12), but the description is repeated here for convenience. All of the sera in one patient's series of sera were tested at one time with one hyaluronidase preparation. Twofold serial dilution, beginning with 1:2, was made for each serum tested. Twelve dilutions, each in the volume of 0.5 cc. in tubes 100 mm. × 13 mm., were made for each serum. The constant amount of hyaluronidase in the volume of 0.25 cc. was added to each serum dilution, and to a control tube containing 0.5 cc. of proteose peptone saline with no serum. The final dilution of serum in the first tube was 1:3, and the final volume in each tube was 0.75 cc. These tubes were mixed by shaking the tube rack, then incubated at room temperature (25° C.) for 20 minutes.

Residual hyaluronidase activity which remained after incubation of the serum dilution and hyaluronidase mixtures was determined as follows:

(1) 1.0 cc. of substrate was added to each tube. This substrate contained equal volumes of three components: dialysed tryptic digest of umbilical cord which contained approximately 0.20% hyaluronic acid (17), normal horse serum (Lederle) which exhibited neither hyaluronidase nor antihyaluronidase activity, and distilled water.

(2) The tubes were mixed by shaking the tube rack, then incubated in a water bath at 37° C. for 30 minutes, and then cooled in an ice water bath for five minutes.

(3) 1.0 cc. of 5% glacial acetic acid was added to each tube, and each tube was shaken individually and read for formation of the mucoprotein clot. The highest dilution of a given serum which exhibited either heavy or thready clot was taken as the antihyaluronidase titer of that serum. Results were expressed in terms of dilution of serum prior to addition of the substrate. Activity of the constant amount of hyaluronidase was demonstrated in the control tube by prevention of the clot.

This mucoprotein clot-prevention test permitted a twofold chance variation of antihyaluronidase titer, but a fourfold chance variation did not occur (12).

RESULTS

The antihyaluronidase responses obtained in three patients with pneumonia and bacteremia are

⁵ These dilutions represent essentially equivalent hyaluronidase activity within the accuracy of the test.

TABLE I

Antihyaluronidase titers of serial sera as determined by titration of the sera against the various hyaluronidases; patients with pneumococcic pneumonia and bacteremia

Type 25 Pn. pneumonia

Hyaluronidases tested	Dates of sera			Multiple change
	11/25/46	12/2/46	12/16/46	
Pn. 1	48	192	768	× 16
Pn. 2	48	192	384	× 8
Pn. 7	48	96	384	× 8
Staph.	6	3	6	× 1
Strep.	3	∠3	∠3	× ∠1
Welch.	∠3	—	∠3	× 1

Type 3 Pn. pneumonia

Hyaluronidases tested	Dates of sera				Multiple change
	3/22/47	3/28/47	4/4/47	4/11/47	
Pn. 1	96	384	1,536	1,536	× 16
Pn. 2	192	384	768	768	× 4
Pn. 7	96	192	768	768	× 8
Staph.	∠3	∠3	∠3	—	× 1
Strep.	3	3	∠3	—	× ∠1
Welch.	∠3	3	∠3	—	× 7

Type 12 Pn. pneumonia

Hyaluronidases tested	Dates of sera				Multiple change
	4/8/47	4/15/47	4/25/47	5/8/47	
Pn. 1	96	384	1,536	1,536	× 16
Pn. 2	96	192	192	768	× 8
Pn. 7	48	96	192	384	× 8
Staph.	3	∠3	∠3	∠3	× ∠1
Welch.	—	—	∠3	∠3	× 1

illustrated in Table I. The antihyaluronidase titer is expressed as the reciprocal of the highest dilution of patients' serum which inactivated the test strength of the hyaluronidase used in the titration. The sera of all of these patients exhibited eightfold or greater rises of antihyaluronidase titer as tested with the three pneumococcus hyaluronidases except that the patient with Type 3 pneumonia exhibited only a fourfold rise of titer against Type 2 Pneumococcus hyaluronidase. However, these sera exhibited no rise of titer as tested with other hyaluronidases. Sera of the first two patients exhibited no rise of titer with the staphylococcus, streptococcus, and Cl. Welchii hyaluronidases, and sera of the third patient exhibited no rise of titer as tested with staphylococcus hyaluronidase. The third patient's last two sera exhibited no antagonism of Cl. Welchii hyaluronidase.

TABLE II

Antihyaluronidase titers of serial sera as determined by titration of the sera against the various hyaluronidases; patients with pneumococcic empyema and bacteremia

Type 1 Pn. empyema

Hyaluronidases tested	Dates of sera						Multiple change
	12/19/44	12/31/44	1/3/45	1/18/45	1/31/45	2/21/45	
Pn. 1	12	768	—	—	384	192	× 64
Pn. 2	3	768	—	—	384	192	× 256
Pn. 7	∠3	192	—	—	192	96	× 64
Welch.	∠3	—	∠3	∠3	—	—	× 1

	12/8/44	1/4/45	1/18/45	
Pn. 1	6	48	48	×8
Pn. 2	12	24	48	×4
Pn. 7	∠3	—	24	×8
Welch.	∠3	∠3	∠3	×1

	4/10/47	4/18/47	4/25/47	5/6/47	5/16/47	6/10/47	
Pn. 1	48	96	384	768	768	768	×16
Pn. 2	24	96	192	768	768	384	×32
Pn. 7	24	48	192	384	384	192	×16
Staph.	12	3	24	6	6	12	× 2
Strep.	—	—	—	∠3	∠3	∠3	× 1
Welch.	—	—	—	∠3	∠3	∠3	× 1

The antihyaluronidase responses obtained in three patients with empyema and bacteremia are illustrated in Table II. Sera of the first patient exhibited rises of antihyaluronidase titer which were 64-fold or greater as tested with the three pneumococcus hyaluronidases, but no antagonism

TABLE III

Antihyaluronidase titers of serial sera as determined by titration of the sera against the various hyaluronidases; pneumococcic endocarditis patient and staphylococcus bacteremia patient

Type 12 Pn. aortic endocarditis with
Type 12 Pn. bacteremia

Hyaluronidases tested	Dates of sera				Multiple change
	10/20/47	10/29/47	11/12/47	11/26/47	
Pn. 2	3	24	192	48	×64
Staph.	24	24	—	24	× 1
Strep.	∠3	∠3	∠3	∠3	× 1
Welch.	∠3	∠3	∠3	∠3	× 1

Hemolytic staphylococcus aureus bacteremia with gastric ulcer

Hyaluronidases tested	Dates of sera						Multiple change
	10/21/47	10/29/47	11/5/47	11/10/47	11/21/47	12/13/47	
Pn. 2	3	6	6	3	6	—	×2
Staph.	3	6	—	12	24	—	×8
Strep.	—	6	—	—	—	6	×1
Welch.	∠3	∠3	—	∠3	—	—	×1

of Cl. Welchii hyaluronidase. Sera of the second patient exhibited fourfold to eightfold rises of titer as tested with the three pneumococcus hyaluronidases, but no antagonism of Cl. Welchii hyaluronidase. Sera of the third patient exhibited 16-fold to 32-fold rises of titer as tested with the three pneumococcus hyaluronidases, with some fluctuation but no definite rise in titer as tested with staphylococcus hyaluronidase. This third patient's last three sera exhibited no antagonism of the streptococcus or Cl. Welchii hyaluronidase.

The antihyaluronidase responses obtained in two other patients are shown in Table III. The first of these patients had Type 12 pneumococcic aortic endocarditis with bacteremia and the second patient had Hemolytic Staphylococcus aureus bacteremia complicating gastric ulcer. Sera of the endocarditis patient exhibited 64-fold rise of antihyaluronidase titer as tested with the Type 2 Pneumococcus hyaluronidase, but no rise of titer as tested with the staphylococcus, streptococcus and Cl. Welchii hyaluronidases. Sera of the patient with staphylococcus bacteremia exhibited eightfold rise of antihyaluronidase titer as tested with the staphylococcus hyaluronidase, elaborated by the organism cultured from this patient's blood, but no significant rise of titer as tested with the pneumococcus and Cl. Welchii hyaluronidases. Too much time elapsed between the two sera which were tested with streptococcus hyaluronidase so that no conclusion can be drawn, but the results suggest that there was no rise in titer of antihyaluronidase antagonistic to the streptococcus hyaluronidase.

The antihyaluronidase responses obtained in two patients with repeatedly positive blood culture due to gram negative bacilli are illustrated in Table IV. The first patient had typhoid fever^a which began about October 7, 1947, and the second patient had B. coli bacteremia of undetermined origin with onset of illness about October 2, 1947. Sera of both of these patients exhibited no rise of antihyaluronidase titer as tested with Type 2 Pneumococcus, staphylococcus, streptococcus and Cl. Welchii hyaluronidases. The second patient's sera exhibited an unusually high titer of antihyaluronidase as tested with Cl. Welchii hyaluronidase, for which no explanation is apparent.

^a Typhoid fever from two different blood cultures of this patient failed to elaborate hyaluronidase in the hyaluronate broth medium.

TABLE IV

Antihyaluronidase titers of serial sera as determined by titration of the sera against the various hyaluronidases; patients with repeated bacteremia due to gram negative bacilli

Typhoid fever with *B. typhosus* bacteremia*

Hyaluronidases tested	Dates of sera				Multiple change
	10/21/47	10/30/47	11/11/47	12/1/47	
Pn. 2	6	—	6	6	×1
Staph.	∠3	—	∠3	∠3	×1
Strep.	—	∠3	∠3	∠3	×1
Welch.	—	12	12	12	×1

B. Coli communis bacteremia of undetermined origin†

Hyaluronidases tested	Dates of sera				Multiple change
	10/16/47	10/20/47	10/28/47	11/20/47	
Pn. 2	6	3	3	3	×½
Staph.	∠3	—	∠3	∠3	×1
Strep.	—	∠3	∠3	—	×1
Welch.	96	96	48	—	×½

* This patient had blood cultures positive for *B. typhosus* on 10/14/47, 10/16/47, 10/17/47, 10/30/47, 11/3/47, 11/13/47, 11/14/47, and 11/18/47.

† This patient had blood cultures positive for *B. coli communis* twice on 10/14/47, and once each on 12/13/47, 12/26/47, 12/30/47, and 2/5/48. The diagnosis was possible endocarditis, but the patient died on February 7, 1948, and autopsy was not obtained.

The antihyaluronidase responses obtained in three patients with pneumococcus bacteremia are illustrated in Table V. Sera of the first two patients exhibited eightfold to 16-fold rises of antihyaluronidase titer and sera of the third patient exhibited fourfold to eightfold rises of antihyaluronidase as tested with the three pneumococcus hyaluronidases, but no rise in titer as tested with the purified bovine testicular hyaluronidase.

DISCUSSION

The specificity of immune human serum antihyaluronidase reported here is in agreement with the specificity of immune rabbit serum antihyaluronidase reported by Duran-Reynals (7); Hobby, Dawson, Meyer and Chaffee (8); McClean and Hale (9); and McClean (10, 11). The lack of specificity for pneumococcus type exhibited by the antihyaluronidase response in patients with pneumococcus bacteremia has been reported previously (12-14).

These findings agree in principle with those of Friou and Wenner (18) on the specific inhibition

of hemolytic streptococcus hyaluronidase by the serum of patients with hemolytic streptococcus infection, but do not support their impression that the streptococcus hyaluronidase is the only hyaluronidase which may be specifically inhibited by human serum.

TABLE V

Antihyaluronidase titers of serial sera as determined by titration of the sera against the hyaluronidases of pneumococcus and bovine testis; patients with pneumococcus bacteremia

Type 14 Pn. pneumonia

Hyaluronidases tested	Dates of sera				Multiple change
	4/7/47	4/15/47	4/22/47	5/6/47	
Pn. 1	96	384	768	768	× 8
Pn. 2	48	384	384	768	×16
Pn. 7	48	384	384	768	×16
Testis	3	∠3	—	∠3	× ∠1

Type 11 Pn. empyema with mesothelioma of pleura

Hyaluronidases tested	Dates of sera		Multiple change
	4/19/47	4/25/47	
Pn. 1	96	1,536	×16
Pn. 2	96	768	× 8
Pn. 7	48	1,536	×32
Testis	∠3	∠3	× 1

Type 5 Pn. empyema

Hyaluronidases tested	Dates of sera		Multiple change
	3/21/47	4/4/47	
Pn. 1	384	1,536	× 4
Pn. 2	192	768	× 4
Pn. 7	192	1,536	× 8
Testis	3	∠3	× ∠1

The immune serum antihyaluronidase reported here in patients with pneumococcus bacteremia has been reported previously to be thermostable; it is unaffected by heating at 56° C. for one hour in a water bath (12). The hyaluronidase inhibitor of normal, non-immune serum has been reported to be thermolabile (3, 5, 6). This study provides no evidence as to whether or not the hyaluronidase inhibitor of normal, non-immune serum specifically antagonizes a particular hyaluronidase.

SUMMARY

Series of sera from patients who suffered bacteremia due to pneumococcus, staphylococcus, *B. typhosus*, and *B. Coli communis*, were tested for

antihyaluronidase against equivalent amounts of pneumococcus, staphylococcus, *beta* Hemolytic Streptococcus, Cl. Welchii, and purified bovine testicular hyaluronidase. Patients with pneumococcus bacteremia exhibited rises in titer of serum antihyaluronidase antagonistic to pneumococcus hyaluronidase, but no rise in titer of antihyaluronidase antagonistic to the other hyaluronidases. One patient with Hemolytic Staphylococcus aureus bacteremia exhibited rise in titer of serum antihyaluronidase antagonistic to hyaluronidase elaborated by the same staphylococcus, but no rise in titer of antihyaluronidase antagonistic to pneumococcus and Cl. Welchii hyaluronidases.

CONCLUSION

In human beings immunized by an infection which elaborates hyaluronidase the immune serum antihyaluronidase is specifically antagonistic to the hyaluronidase of the particular species of organism from which the antigen was derived, so far as the pneumococcus is concerned. This finding probably applies to the staphylococcus, and to other species of organisms which elaborate hyaluronidase.

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RENAL AND CIRCULATORY FACTORS IN THE EDEMA FORMATION OF CONGESTIVE HEART FAILURE¹

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While many contributions have been made to the study of heart failure, the basic factor initiating the chain of events leading to edema formation in that condition remains unidentified. It is hoped that this report will present evidence which may prove useful in elucidating the problem.

METHODS

All renal and circulatory studies to be reported were done on cardiac patients. Twenty-one patients were in heart failure at the time of the observations, while 18 were compensated cardiacs. In ten cases, studies were made in the same individual during failure and again after compensation had been restored. Patients were treated with digitalis, diuretics, bed-rest, and a low salt diet which, in actual ward experience, has not produced a very rigid restriction of sodium. Some patients had been on maintenance doses of digitalis prior to admission to the hospital. In these cases, digitalis was continued while other medication was withheld until initial studies were completed. When compensation was restored, as indicated by reduction of dyspnea and edema and maintenance of steady weight, all diuretic medication was withheld for at least 48 hours (in most cases for a much longer time), and the determinations were repeated.

The cardiac output was determined by the Fick principle, utilizing the technique of right heart catheterization (1). This involved estimation of arterial and mixed venous blood oxygen content, and oxygen consumption. From the oxygen content of arterial (A) and venous (V) blood, the approximate oxygen saturation of the mixed venous blood was calculated from the relation $V/A \times 100$. This tacitly assumes complete arterial saturation—which is near enough the truth for our purposes. Intracardiac pressures were measured by an optical manometer (2). Blood volume was measured with the dye T-1824 (3). The method used differed from that most frequently described in that the relation between dye dilution and optical density (at 625 m μ as measured in the Beckman spectrophotometer) was determined directly in the blood of the subject of each experiment. Standards were made up by diluting known amounts of the injected dye solution in whole blood, and the blood volume was calculated from these measurements, as compared with

the optical density of a sample drawn ten minutes after dye injection. This procedure avoids the controversial errors inherent in the centrifuge hematocrit. Plasma volumes were not measured, because these are less constant than blood volume since they vary inversely with cell volume. Plasma volumes are a less direct measure of the fluid which distends the vascular tree and brings about some of the signs of congestive failure, than are blood volumes. The thiocyanate space was estimated by the method described by Crandall and Anderson (4) and modified by Gregersen and Stewart (5). Glomerular filtration rate was measured by the sodium thiosulfate clearance (6). Sodium was determined by the method of Bradbury (7). Since the sodium levels in the plasma were quite constant, the sodium excretion in milligrams can readily be estimated by multiplying the clearance figure by 3.22. Sodium clearances thus measured were the response of the kidneys to a rather large sodium load. This sodium load was the infusion of a little less than 500 cc. of 1.6% sodium thiosulfate. A 15-minute period for equilibration was allowed before starting the clearance test. At this time the level of serum sodium was found to have returned to the control value. Because of various technical difficulties, all the above observations were not always obtained in a given case. When body size corrections were made, the trends described below were not changed. Weight changes during an experiment were large, and it was decided to record the figures on an individual rather than on a body size basis.

RESULTS

Figure 1 shows the relation of the filtration rate to the sodium resorbed, and to the sodium clearance, in uncompensated and compensated individuals. The points indicating sodium resorbed correlate very closely with filtration rate (8), but neither filtration rate nor the amount of sodium resorbed shows any consistent change which is related to whether the patient is compensated or uncompensated. The ability to excrete sodium, on the other hand, consistently improves as the patient regains compensation.

Thiocyanate space is definitely greater in uncompensated than compensated patients (Table I). The kidneys of the compensated patients are able

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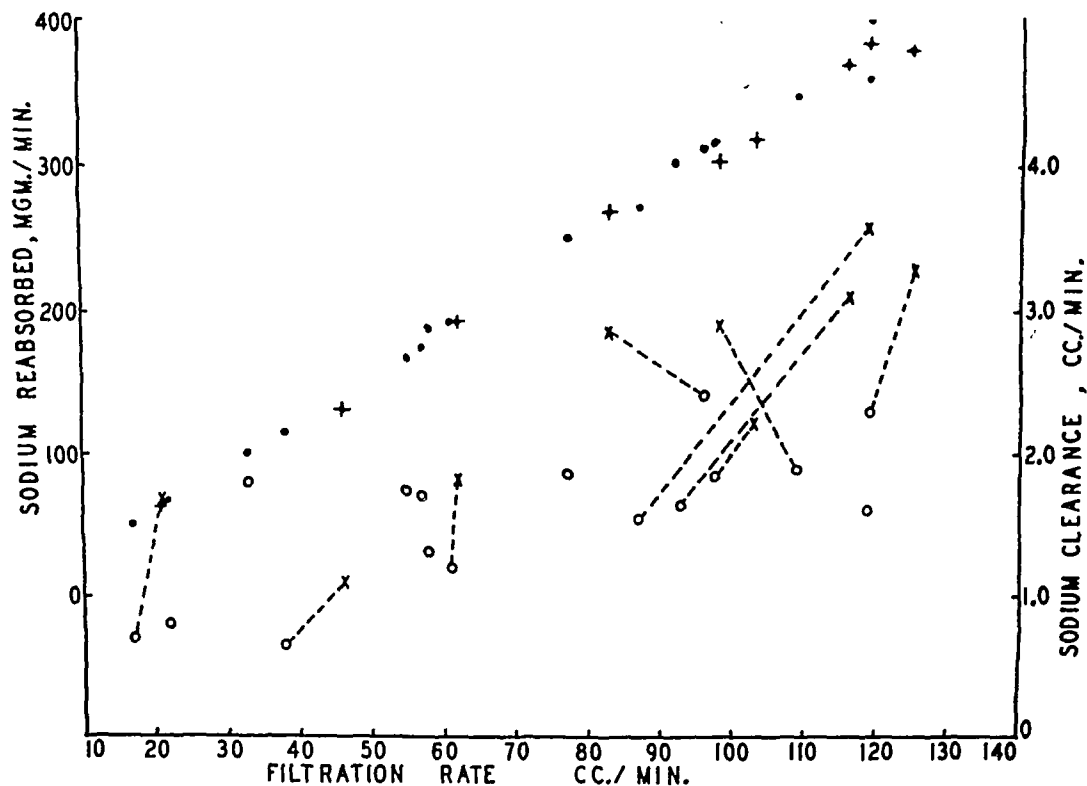


FIG. 1. THE RELATIONS BETWEEN SODIUM REABSORBED AND FILTRATION RATE IN UNCOMPENSATED (•) AND RECOMPENSATED (+) PATIENTS, AND THE RELATION BETWEEN SODIUM CLEARANCE AND FILTRATION RATE IN UNCOMPENSATED (o) AND RECOMPENSATED PATIENTS (x)

Dotted lines connect determinations on the same patient.

to excrete sodium at nearly twice the rate of those of the uncompensated patients. Regaining the ability to excrete sodium goes hand in hand with the reduction in thiocyanate space (Figure 2). The standard errors (Table I) are large because of the natural variability of the data. The increase seen in each case with recompensation (Figure 2) indicates that there is a significant change in the ability to excrete sodium. No relationship could

be found between filtration rate and thiocyanate space.

Blood volume changes, unlike those of thiocyanate space, were inconsistent, and the averages for the two groups are not different (Table I). The presence of inanition, on entrance, in many cases, no doubt played a complicating role in the blood volume changes. The average arterial oxygen content, for example, is definitely lower in un-

TABLE I
Renal and circulatory data from a series of uncompensated and recompensated cardiac patients

Type of patients		Sodium thiocyanate space	Blood volume	Serum sodium	Filtration rate	Sodium clearance	Right ventricle filling pressure	Oxygen consumption	Arterial blood oxygen	A-V oxygen difference	Cardiac output	Venous oxygen saturation
		L.	L.	mgm./100 cc.	cc./min.	cc./min.	mm. Hg	cc./min.	cols./100 cc.	cols./100 cc.	L./min.	per cent
Uncompensated	No.	14	12	14	14	14	13	17	16	14	14	14
	Mean	27.8	5.30	324	70.0	1.35	15	261	15.60	7.3	3.8	55
	S. E.	1.9	.33	2	9.2	.13	3	15	1.00	0.5	0.4	2
Recompensated	No.	9	10	10	10	9	6	11	11	11	11	11
	Mean	20.2	5.27	319	81.2	2.31	9	252	17.52	5.7	4.7	67
	S. E.	1.5	.30	4	11.0	.29	2	6	0.80	0.3	0.4	1

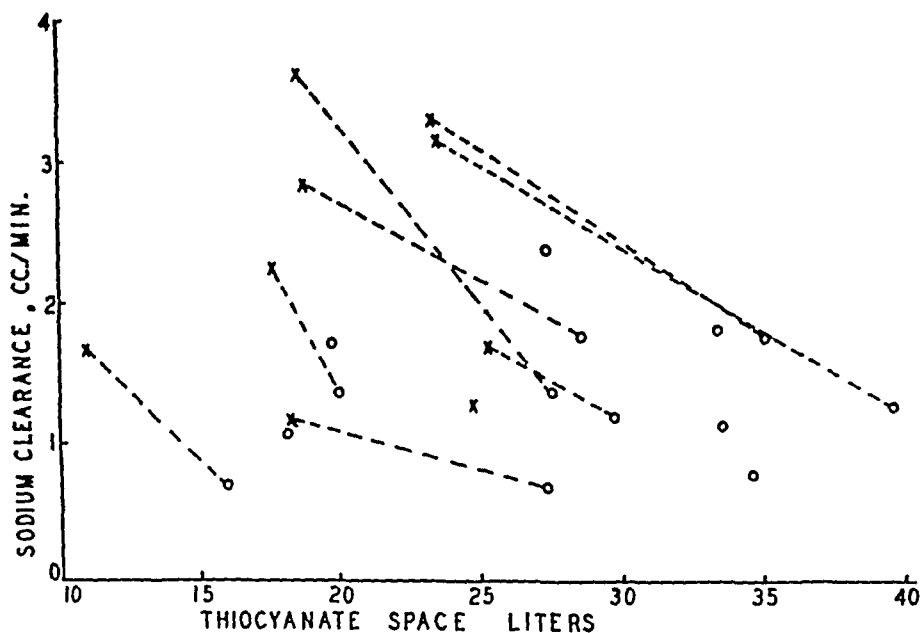


FIG. 2. THE RELATION BETWEEN THIOCYANATE SPACE AND SODIUM CLEARANCE IN UNCOMPENSATED (o) AND RECOMPENSATED (x) CASES
Dotted lines connect determinations in the same subject.

compensated than in compensated cases. Favorable changes in nutrition and water balance, and, in extreme anemias, transfusions, can account for the hemoglobin increase on compensation.

The filling pressures of the right ventricle were higher in uncompensated than in recompensated cases (Table I). The pressure did not always decrease as compensation was regained, nor was there any definite correlation between pressure height and the thiocyanate space, as might be expected if the one were the primary factor leading to an increase in the other. There was little statistical significance in the difference between the means of the pressures in compensated and recompensated cases.

The cardiac output values for the uncompensated group are placed in two categories. Two of 16 cases showed failure in the presence of an abnormally high output, and with unusually high mixed venous blood oxygen content. One of these patients probably had beri-beri, while the other failed from unknown causes. Only one of these patients could be studied after compensation was restored, and he showed a fall in cardiac output, thiocyanate space, blood volume and right heart filling pressure, with an increase in sodium clearance. Our knowledge of congestive heart failure is too inadequate to allow successful integration of these two unusual cases into the picture as a whole. It should

also be pointed out that in both these cases, the venous sample was obtained from the right atrium, so that incomplete mixing may have contributed to the high venous oxygen content.

Even with these two cases included, the average output is higher in the compensated than in uncompensated cases. There is, however, considerable overlap (Figure 3), and compensation is not necessarily accompanied by an increase in output. Excluding the high output failures (Table I),

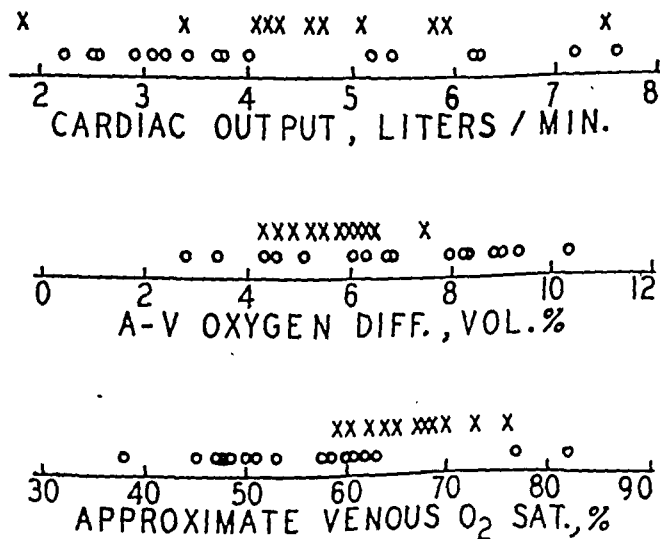


FIG. 3. DISTRIBUTION OF CARDIAC OUTPUT, A-V OXYGEN DIFFERENCE, AND APPROXIMATE OXYGEN SATURATION OF MIXED VENOUS BLOOD, IN UNCOMPENSATED (o) AND RECOMPENSATED (x) CASES

the means of the uncompensated and recompensated cases differed by an amount that is significant at the 10% level.

The arterio-venous oxygen difference is definitely larger in the uncompensated cases than it is in the compensated cases. The difference of the two means is significant at better than the 3% level, but there is considerable overlap between the two groups and the change on compensation of a given individual was not always consistent.

The approximate venous oxygen saturation increased consistently as compensation was regained. Statistically the difference between the means of the uncompensated and recompensated groups was highly significant ($P < 0.001$).

DISCUSSION

During recent years attention has been focused on the kidneys as playing an important role in the edema formation of congestive heart failure (8-15). The underlying mechanism by which the failing circulation causes the kidney to excrete sodium and water in inadequate amounts has been the subject of considerable speculation. However, it would seem to be well as a preliminary step to inquire into the manner in which renal function is disturbed, before discussing the mechanism of this disturbance.

It is, of course, difficult to decide whether the renal dysfunction seen in congestive failure is due to renal impairment or to circulatory disturbance. None of the patients were uremic and most of them were capable of secreting urine whose specific gravity was above 1.02; but the low thiosulfate clearance in the compensated state is, itself, good evidence of renal impairment.

Warren and Stead (12) pointed to the diminished renal blood flow which they noted in congestive heart failure. Merrill (9), following out this concept of "forward failure," found a decreased filtration rate in patients with congestive heart failure as compared to normal, and so concluded that the diminished excretion of sodium came about simply because a smaller amount was filtered. Mokotoff *et al.* (8) have supported this view with the further observation that sodium resorbed varied directly with filtration rate, and that the latter was less in cardiacs than in normal individuals.

One should not attribute much significance to the fact that filtration rate and resorbed sodium are proportional. It should be remembered that relatively huge quantities of salt and water are filtered and reabsorbed, even by a handicapped kidney, in comparison to the small amounts excreted. This rather close correlation between filtration rate and sodium resorption is explained by the manner in which the figures are derived. The amount of sodium resorbed is calculated by subtracting the quantity of sodium eliminated in the urine from the amount filtered. The urinary sodium is a relatively small quantity, which causes a negligible diminution in the figure for the amount filtered. The sodium filtered is obtained by multiplying the plasma sodium level by the filtration rate. The plasma sodium is held within narrow limits. Thus plotting sodium resorbed against filtration rate is not unlike plotting filtration rate against filtration rate times a constant, inevitably giving the picture seen in Figure 1 ($\bullet +$).

Our data confirm the findings of Warren and Stead (12), of Merrill (9) and of Mokotoff and his collaborators (8) that the filtration rate in cardiacs is much less than it is in normal individuals. It must be emphasized, however, that patients can regain clinical compensation without in any significant degree increasing their filtration rate.

These findings in relation to filtration rate coincide with those of Seymour *et al.* (15) who studied six patients in congestive failure. They found that as the patients regained compensation the filtration rate was decreased in one and increased in four, but in only one of these was the increase significant in amount. Thus the filtration rate has an ambiguous relation to the state of compensation in cardiac patients. There is a widely varying amount of sodium filtered and consequently a widely varying amount of sodium presented to the tubules of the several patients. The amount of sodium presented varies over a range which is nearly equal whether the patient is uncompensated or recompensated, and since it is nearly all resorbed, there is a similar wide variation in the amount of sodium reabsorbed.

The ability to excrete sodium, on the other hand, always increased after compensation had been regained. The experiments showing this (Figures 1 and 2) are the more conclusive in that the sodium clearance is higher after compensation in spite of

the fact that the patients had been on salt restriction and had received the same test load of sodium as they received when they were admitted in the uncompensated state. The total sodium load was greater therefore when they were uncompensated and the sodium clearance was smaller.

From the mean figures of Table I, it may be calculated that the over-all rise in filtration of sodium was about 36 mgm. per minute, which is sufficient to account for the increase in sodium excretion of about 3.1 mgm. per minute. But, it is not necessary to assume, as some workers have, that the extra excretion is due to extra filtration. For, in the first place, if the extra sodium filtered were resorbed to the usual extent of about 99%, then only about 0.3 mgm. extra sodium per minute would have been excreted. Secondly, extra sodium is excreted in some cases without an increase in filtration. And thirdly, a seemingly minor variation in per cent sodium resorption may represent a great variation in sodium excretion, involving several grams per day, and, therefore, possibly large volumes of excreted water. Some factor connected with the state of uncompensation may force the kidney to resorb sodium and water not necessarily in greater amount but more *completely*.

It is reasonable to suppose that this factor is related to the circulatory states of the patients. Of the various circulatory findings which we have compared there is an improvement in oxygen capacity as measured by arterial oxygen content, in cardiac output and in A-V difference. As seen above, changes in these categories are not so consistent as are changes in oxygen saturation of the mixed venous blood. Here there is a good distinction between compensated and uncompensated cases. A lowered venous oxygen saturation appears to be a definite part of the usual picture of congestive heart failure, and is a deficiency which was corrected in the process of recovery in all of our patients. This implies that during failure the cells of the body are subjected to an environment having an abnormally low oxygen concentration—a true hypoxia. In extracting oxygen from this medium to carry on vital processes, the tissues of the uncompensated cardiac are at a disadvantage, suggesting a handicap to normal cellular activity.

The oxygen saturation of the mixed venous blood represents the conditions under which the tissues are working. To illustrate, let us use the

average figures for per cent oxygen saturation of the venous blood. This average for the uncompensated group is 53%, which, when applied to the oxygen dissociation curve, represents an oxygen tension of 28 mm. The figure of about 67% in compensation gives a venous oxygen tension of about 35 mm. This ignores differences in carbon dioxide tension between the two groups, which would narrow the differences slightly. From this, applying Henry's law, it appears that the fluid medium surrounding the tissue cells contains a lower concentration of oxygen in heart failure than otherwise.

The organism, threatened with hypoxia, may react in several different ways. The oxygen consumption may fall as is the case in the children afflicted with congenital heart disease of the cyanotic group (16). The cardiac output may rise as is the case in severe anemia and hyperthyroidism. The hemoglobin may increase as is the case at high altitudes or in congenital heart disease. Another mechanism may be available, which usually ends in a vicious cycle. The kidney may conserve water and salt. Increased tubular reabsorption is seen as a result of afferent nerve stimulation and exercise (17). In response to a short-lasting episode this may help in conserving the blood volume and in increasing the circulation rate; as a chronic response it may lead to edema and congestive failure of the circulation.

Our patients in the uncompensated condition showed a lowered cardiac output, a reduced hemoglobin and an increased oxygen consumption. The increase in the oxygen consumption is probably due to dyspnea, restlessness, and other discomforts attendant upon the disease. These would tend to increase the cardiac output. The weakened heart can only partially meet this demand for more blood, so that the A-V differences rise, and the oxygen saturation of the mixed venous blood falls.

Lowered hemoglobin has a similar effect upon the cardiac output and venous oxygen saturation, but not upon the A-V difference. For example, one patient had an arterial oxygen of 8.20 vol. %. His venous oxygen was 3.15 vol. %, giving a relatively low A-V difference and relatively high cardiac output. His approximate venous oxygen saturation was 38%, a very low figure. On compensation, and with a higher hemoglobin, the cardiac output and A-V difference did not change for

the better but the venous oxygen saturation was raised to a figure representative of the compensated group. Anemia may give rise to oxygen saturation of the mixed venous blood that is well below the figure of 60%. Thus Brannon *et al.* (18) report data in which four of their cases have saturations between 40 and 50% with no mention of uncompensation. It is recognized that anemia predisposes to dyspnea and edema even when the cardiac output is high. Studies of renal physiology in such cases will be of great interest.

It should be mentioned that Fahr and Ershler (19) deny the importance of anoxia in the production of edema on the ground that congenital heart cases of the cyanotic group may not have edema even with a low arterial oxygen saturation. The tremendous increase in hemoglobin seen in these cases prevents the venous blood from becoming extremely unsaturated. The figures in so far as they are obtainable indicate that the saturation of the mixed venous blood is within normal limits or on the border line. Thus in two cases of Tetralogy of Fallot reported by Dexter *et al.* (20) the oxygen saturation of right atrial blood was 73% and 59%, while that in one such case observed by us was 60%. Figure 3 shows that the dividing line between cardiacs with and without edema falls at about 60% saturation of the mixed venous blood. Therefore there is no reason to expect such patients to show edema on the basis of their hypoxia.

The well-recognized benefit from the administration of oxygen in congestive failure with obstruction anoxia from pulmonary edema is also pertinent. Barack and Richards (21) have shown that oxygen administration will diminish edema which may return on cessation of the treatment.

It is conceivable that anoxia might increase the permeability of the capillaries so that there is a local production of edema by the filtration of a plasma-like fluid into the tissue space. The fact, however, that the protein content of the edema fluid in cardiac cases is very low leads to the conclusion that a normal capillary barrier is maintained in congestive failure.

It seems more likely that the edema is formed from ingested water which cannot get out through the kidneys. It is well known that water, administered by vein or by mouth, will be stored in the extravascular spaces until the kidney removes it. Its removal is brought about by lessening the

reabsorptive capacity of the kidney. The inability of the kidney in congestive failure to get rid of edema fluid, we believe, is due to an overworking of the mechanism which reabsorbs salt and water, and this in turn seems to be closely related to cellular hypoxia.

The manner in which cellular hypoxia causes more complete tubular reabsorption and leads to the edema is difficult to understand. The idea that changes in renal activity are brought about by the direct effect of anoxia upon the kidney is probably ruled out by the fact that the oxygen tension of renal vein blood in congestive failure may be within normal limits (9). With reduction in blood flow the oxygen consumption must be reduced or the A-V difference increased. In any case an abnormality of renal function is evident.

It seems likely that hormonal influences may play a role in effecting a more complete tubular reabsorption. Fremont-Smith (10) postulated an increased "antidiuretic factor" which prevents, in edematous patients, the diuresis which normally follows the ingestion of water. Taking water, in these cases, causes a greater than normal blood dilution. The source of such an antidiuretic principle may be the posterior pituitary gland (22), which is known to secrete an antidiuretic hormone in times of stress (17).

Numerous observers have assessed the effect of adreno-cortical steroids and similar substances in increasing the action of the kidney to reabsorb sodium and water. Edema may be produced as a result of overdosage of desoxycorticosterone, or sex hormones (23, 24).

The classical idea that an excess of hydrostatic over osmotic pressure in the systemic capillaries is a primary factor in the production of cardiac edema does not receive support from these observations. Right auricular pressure, though higher than normal, was not consistently higher in the uncompensated cases than it was in the compensated cases. Moreover Altschule (25) presents a series of cases of cardiac edema in which the venous pressure is within normal limits. He also reviews many such cases from the literature.

It is quite probable that once failure is established, increased venous pressure (resulting in increased capillary hydrostatic pressure) and lowered plasma proteins, which are seen in many cases

of congestive failure, contribute to the persistence and perhaps to the increase of the edema.

The evidence presented above, however, indicates that the circulatory disturbance produces a reduced oxygen tension in the mixed venous blood, and that this brings about in some way a more complete resorption of salt and water by the kidney, which in turn is responsible for the edema.

SUMMARY

1. Studies are reported on 21 patients during congestive heart failure, and on 18 patients with heart disease but no signs of failure. In ten individuals duplicate studies were obtained both before and after compensation was restored.

2. These data do not support the theory that edema fluid is eliminated in these patients by increased filtration in the kidney. Rather they point to a less complete tubular resorption of sodium and water as the important factor.

3. On the basis of our circulatory data, the uncompensated patients were most clearly distinguished from the recompensated patients by their lowered mixed venous oxygen tension, suggesting that this handicap to cellular respiration is related to the symptomatology of congestive heart failure.

4. That the reduced sodium clearance, increased thiocyanate space, and lowered venous oxygen tension, in failure, are causally related is suggested, and possible mechanisms of their inter-relation are discussed.

5. Secondary factors which may contribute to the edema in a given case of congestive heart failure have been briefly mentioned.

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UROPEPSIN EXCRETION BY MAN. I. THE SOURCE, PROPERTIES AND ASSAY OF UROPEPSIN^{1,2}

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The conventional method used to study gastric secretion and activity involves the withdrawal and examination of specimens of gastric juice. Such a procedure subjects the average patient to considerable physical and emotional stresses. Further, the repeated collection of samples of gastric juice from a great number of individuals over a prolonged period of time presents many practical difficulties. It would, therefore, be advantageous to possess a means for the study of gastric activity which would have none of the disadvantages involved in the withdrawal of the gastric contents. During the course of studies designed to develop some technique for this purpose, we investigated the excretion of uropepsin, a pepsin-like enzyme in the urine.

The presence of an enzyme in the urine, with proteolytic activity at strongly acid reactions, was first demonstrated by Brucke (1). Since that time occasional investigators have reported information concerning the nature of this substance, its source, and the factors controlling its appearance in the urine. Unfortunately much of the early work in this field was accomplished with the aid of methods of a questionable degree of accuracy. In particular this is true for the procedures utilized for the determination of the activity of this substance. Accordingly, much of this work, which has been summarized in an excellent review by Bucher (2), requires confirmation. More recent studies by Farnsworth, Speer and Alt (3) and by Bucher (2), have reopened this field of inquiry and have contributed additional knowledge concerning uropepsin.

Certain facts appear to be well-established. Uropepsin is not found in the urine of gastrectomized

dogs (4), gastrectomized cats (5) or patients with the achylia gastrica of pernicious anemia (3). Presumably, therefore, the function of the peptic cells of the gastric mucosa is essential for the formation of uropepsin. Consequently, it is possible that some correlation might be found to exist between gastric activity and the amount of uropepsin in the urine. Towards that end we considered it desirable to define quantitatively such relationships as could be observed between any aspect of gastric function and the excretion of uropepsin. The studies of Bucher (2) and Farnsworth *et al.* (3) have already noted certain of these correlations. In the present series of studies, an attempt is made to define further certain of the factors that regulate the excretion of uropepsin by man.

METHODS

Methods for the estimation of uropepsin have been described by both Bucher (2) and Farnsworth *et al.* (3). These depend upon incubation of a sample of urine with a denatured hemoglobin substrate (6) over periods of either 60 minutes or 18 hours (3) and the subsequent determination of the extent of proteolysis by the Folin and Ciocalteu procedure (7). Since both the hemoglobin substrate and the urine itself contain substances which react with the Folin-Ciocalteu reagent, the amount of acid-soluble "tyrosine-like" material present prior to incubation was determined after incubation of an heat-inactivated specimen of urine with the substrate. The difference between these two values was utilized as the index of uropepsin activity.

Such methods involve certain objectionable features. In the first place, although Bucher presented data which tended to indicate that the addition of urines of widely different pH to the acid hemoglobin substrate did not lead to inaccurate results, it is not difficult to demonstrate that the addition of 1 ml. of unadjusted urine to 5 ml. of the standard hemoglobin substrate does result in changes in the final pH of the mixture, changes which are dependent on the composition and reaction of the urine specimen itself. The desirability of measurement of enzyme activity at a constant pH is too well known to require further comment. In the second place, although heat-inactivation of urine destroys its uropeptic

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activity, the use of such a specimen as a control may lead to an incorrect estimation of the uropeptic activity since some specimens of urine have been found to undergo an increase in their content of "tyrosine-like" substances on heating. Although this phenomenon has not been noted with either regularity or frequency, its occasional appearance vitiates the accuracy of such a procedure.

In order to circumvent such objections, the following procedure was utilized. An aliquot of 20 ml. of urine was adjusted to pH 1.5 and made up to a total volume of 25 ml. with distilled water. One ml. of the adjusted urine, representing 0.8 ml. of the undiluted urine, was incubated with 5 ml. of the denatured hemoglobin substrate at pH 1.5 as described for the estimation of pepsin (6) except that the temperature during incubation was maintained at 37° C. At the end of the prescribed period of incubation, the reaction was stopped by the addition of trichloroacetic acid and the concentration of acid-soluble "tyrosine-like" substances in the filtrate was determined by the Folin-Ciocalteu method adopted to use of a spectrophotometer. The concentration of chromogenic substances originally present in both urine and substrate was determined by the separate addition of 1 ml. of the adjusted, diluted urine and 5 ml. of the hemoglobin substrate directly to trichloroacetic acid. The difference in readings obtained from the incubated and unincubated samples was taken to indicate the increase in concentration of chromogenic substances due to proteolysis.

RESULTS

It was found possible to shorten materially the times of incubation used by both Bucher and Farnsworth *et al.* With most urines, incubation for only ten minutes produced a significant degree of proteolysis. The use of such a short period of incubation offers definite practical advantages. However, owing to the limits of accuracy of the analytical procedure, changes during incubation of only 2% transmission could fall within the limits of experimental error. Whenever such small changes were obtained, the incubation was repeated for 30 minutes. If the low degree of apparent proteolysis was due solely to the relative inaccuracy of the technic, use of the longer period of incubation failed materially to produce an increase in such values. On the other hand, if the low results obtained from a ten-minute period of incubation were actually due to a low degree of uropepsin activity, values approximately three times as great as those previously found were obtained by means of the 30-minute period of incubation. These higher values were then divided by three in order to reduce them to the basis of a ten-minute period of incubation. The justification for

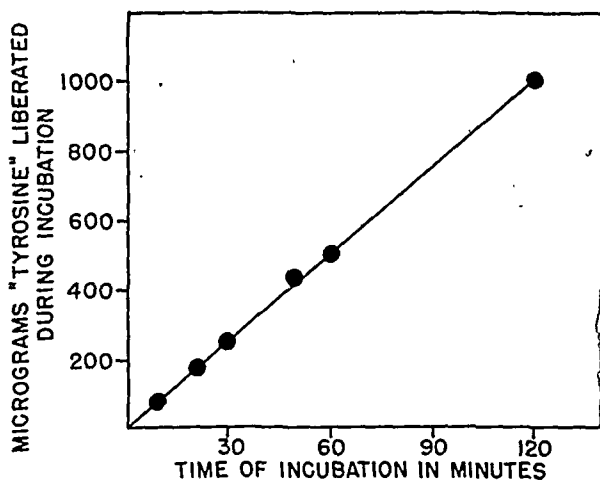


FIG. 1. EFFECT OF INCUBATION TIME ON UROPEPSIN ACTIVITY

The results illustrate the amounts of "tyrosine" released by 1 ml. of adjusted urine during different incubation periods.

such a procedure is illustrated in Figure 1 which demonstrates that the degree of proteolysis produced by uropepsin increases in a linear manner with time throughout a two-hour period of incubation.

In view of the above findings, we have routinely based all of our calculations of activities on a ten-minute period of incubation. For the sake of convenience, an arbitrary unit of activity of uropepsin has been selected, which is defined as that quantity which, during a ten-minute incubation in the standard assay, releases 1 mg. of "tyrosine-like" substance. To derive this unit, the results obtained from the spectrophotometric assay are recalculated in terms of the activity contained in 1 ml. of undiluted urine. For example, if 1 ml. of the adjusted, diluted urine has been found to release 40 γ of "tyrosine" during the ten-minute period of incubation, 50 γ would have been released by each ml. of the undiluted urine. Accordingly, every 20 ml. of urine would contain 1 unit of uropepsin.

In order to proceed with the interpretation of the amount of uropepsin as it is assayed in urine specimens, it is of paramount importance to be able to demonstrate that such activity represents the actual quantity of uropepsin as it was originally excreted in the urine. If the activity undergoes gradual decreases due to the instability of the enzyme or if other substances present in the urine partially inhibit its activity, assays on specimens of

urine may fail to yield accurate data concerning the amount of uropepsin actually excreted by any particular subject. Experiments were therefore undertaken to investigate the problem of the stability of this enzyme and to detect the presence in urine of any inhibitory substances which might mask some fraction of the total activity.

Bucher (2) has demonstrated the stability of uropepsin activity in urine during four-day periods of storage at room temperature and for at least two weeks during standing in the refrigerator. Our own results completely confirm such findings as is illustrated by Table I. Furthermore, the addition of toluol to the urine fails to affect the activity and preserves its stability during storage at both room temperature and in the refrigerator. As a result we have adopted as routine the collection of speci-

TABLE I
Stability of uropepsin

Duration of storage	Conditions of storage		Toluol added	Peptic activity
	Room temp.	Cold room (3°-5° C.)		
days				
0			No	78
0			Yes	74
1	x		Yes	82
1		x	Yes	75
7	x		Yes	78
14	x		Yes	73
28		x	Yes	74

Peptic activity expressed as micrograms "tyrosine" released by 1 ml. of urine during standard assay.

nens of urine in containers to which sufficient toluol had been added and have stored them at ice box temperature (3° - 5° C.) in such instances in which assays could not be performed on the day of their collection. The toluol was always removed prior to assay.

The stability of uropepsin under these conditions has certain practical implications since it has been found feasible to collect specimens of urine in other cities and to have them shipped to our laboratory for assay. For this purpose, a specimen of urine is collected as described, its total volume measured and notation is made of the period of its collection. An aliquot (approximately 25 ml.) is placed in a large test tube, covered with toluol and sent to our laboratory for examination.

Efforts to demonstrate the presence of uropepsin

TABLE II
Effect of dialysis on activity of uropepsin

Urine	γ "Tyrosine" released by 1 ml. urine during standard assay	
	Experiment A	Experiment B
Before dialysis	58	75
After dialysis	51	72

Urines dialyzed overnight against running ice-cold water in Visking membranes. Results after dialysis recalculated to take account of dilution during dialysis.

inhibitors in urine have not been successful. The possible presence of an inhibitor of low molecular weight was ruled out by the results illustrated in Table II which indicate that dialysis of the urine failed to alter its activity, a finding which, at the same time, confirms the presumed protein-like nature of the enzyme itself. Washing with chloroform has also been described as an effective method for the removal of substances that inhibit the activity of some proteolytic enzymes. Such a procedure failed to affect the activity of uropepsin. Furthermore, since active uropepsin is believed to resemble or to be identical with gastric pepsin, substances which inhibit uropepsin should probably also inhibit gastric pepsin. In order to investigate this possibility, the effect of the addition of various specimens of urine on the activity of a solution of gastric pepsin of known potency was determined. Such results are found in Table III and indicate that urine contains no substances that inhibit the activity of pepsin. The lack of inhibitor substances and the stability of uropepsin indicate that the methods used in this study determine the actual amount of uropepsin excreted. The uropep-

TABLE III
Effect of urine on activity of gastric pepsin

Enzyme preparation	Peptic activity of enzyme preparation*	
	Experiment A	Experiment B
Urine alone	87	120
Gastric pepsin† alone	317	401
Urine and gastric pepsin‡	400	541
% Recovery of activity of gastric pepsin in urine	99	104

* Activity expressed in terms of γ "tyrosine" released by 1 ml. enzyme preparation during standard assay.

† A dilute solution of Wilson 1:22,000 pepsin in water.

‡ A solution of the same concentration in urine.

sin assays accordingly offer a valid procedure for the estimation of the amount of uropepsin excreted during a given period of time.

In previous studies on uropepsin, it has been assumed that the proteolytic activity of uropepsin at strongly acid reactions is proof of its pepsin-like nature. Nevertheless, although maximum activity of the enzyme is evident at lower pH, considerable proteolytic activity can be demonstrated when the urine and substrate are incubated at pH 3.5. This latter reaction has been designated (6) as appropriate for detection and determination of catheptic activity. Furthermore, gastric juice is known to contain considerable quantities of enzymes with catheptic activity (8). Inasmuch as uropepsin presumably originates in the stomach, it might be postulated that some, if not all, of uropeptic activity is catheptic rather than peptic in nature.

However, it appears safe to assume that most, if not all, of the proteolytic activity of urine at pH 1.5 may be attributed to the activity of a pepsin-like enzyme since cathepsins derived from animal tissues are known to be rapidly inactivated at this reaction (9) and since we have been able to demonstrate the stability of uropepsin over a 24-hour period at this reaction. A comparison of the pH activity

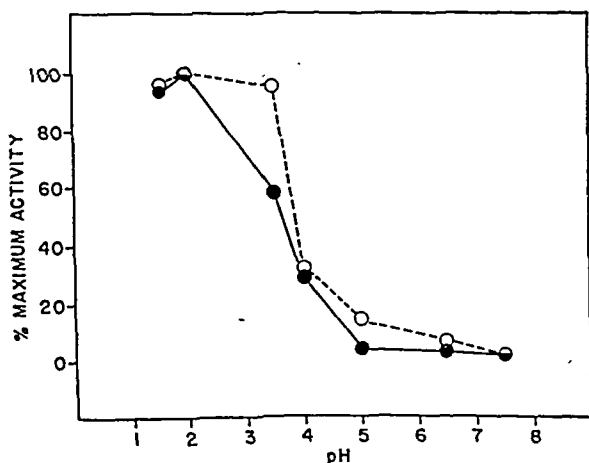


FIG. 2. EFFECT OF pH ON ACTIVITY OF UROPEPSIN AND GASTRIC PEPSIN

Activity calculated in terms of amounts of "tyrosine" released by 1 ml. of urine or aqueous solution of 1:22,000 Wilson pepsin during ten-minute incubation periods. In every case, both enzyme solution and substrate were adjusted to indicated reaction. Closed circles represent gastric pepsin; open circles, urine.

curves of uropepsin and of gastric pepsin is demonstrated graphically in Figure 2. For this purpose the pH activity curve of uropepsin was compared with the pH activity curve of a specimen of gastric pepsin diluted in urine from a patient with pernicious anemia (containing no uropepsin activity). The results reveal certain points of resemblance and of dissimilarity between the two curves. Uropepsin manifests considerably more activity at pH 3.5 than does gastric pepsin. Otherwise the two curves can be almost superimposed upon one another. Whether or not the high degree of proteolytic activity of urine at pH 3.5 can be attributed to the presence of a catheptic enzyme has not yet been determined and must await examination of its action on some of the polypeptides demonstrated by Bergman *et al.* (10) to be useful for the identification of the type of enzyme involved. It would appear unlikely that much of the activity of urine at pH 3.5 could be due to the presence of a catheptic enzyme since the activity decreases so abruptly even at pH 4.0.

By way of comparison, the pH activity curve of the cathepsin of gastric juice should be consulted (11). This activity curve was based on the degree of proteolysis of casein, instead of hemoglobin, and the effect of urine on this curve is unknown. Nevertheless, it would appear safe to conclude that uropepsin and pepsin resemble one another and are markedly dissimilar to gastric cathepsin in regard to their pH activity curves.

It is of interest to note that Freudenberg demonstrated some activity of gastric cathepsin at pH 2 and pointed out that the measurement of peptic activity at this pH cannot be accepted as an accurate method for the determination of the amount of pepsin present in gastric juice (11). Such a warning reemphasizes the importance of measurement of uropepsin at a constant pH, preferably at pH 1.5, particularly if uropepsin is to be interpreted solely as an excretion product of a pepsin-like enzyme.

It is possible to demonstrate that all of the proteolytic activity of urine at pH 3.5 can be attributed to the excretion of a substance produced by the gastric mucosa. This was accomplished by examination of the proteolytic activity of the urine of gastrectomized patients and of patients with pernicious anemia. In every such instance, when no activity was noted at pH 1.5 none was found at pH

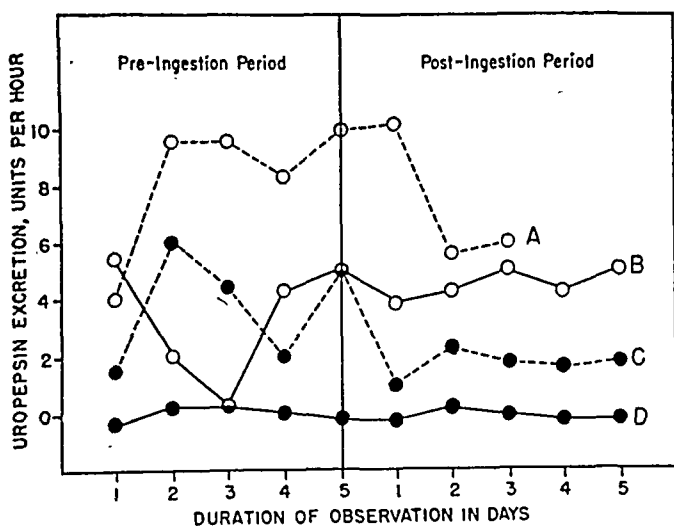


FIG. 3. EFFECT OF ORAL INGESTION OF PEPSIN ON UROPEPSIN EXCRETION

Five grams of scale pepsin U.S.P. ingested by each subject at end of period corresponding to collection of urine for fifth day of observation. Urines were collected in each instance for the entire 24-hour period.

A, B, and C represent uropepsin excretions of three healthy, adult male subjects. D represents uropepsin excretions of a patient with pernicious anemia.

3.5. Whereas such evidence does not constitute conclusive proof, it suggests that the proteolytic activity of urine at pH 3.5 may be due to the presence of the same substance whose activity we determine at pH 1.5 and which is considered to be uropepsin.

Controversies concerning the source of uropepsin have been discussed by Bucher (2). Obviously, if it originates in the stomach, it presumably must be transported to the kidney by the blood

stream. The mode of entrance of the enzyme into the circulation and the form in which it enters the blood are still problematical. It is conceivable that pepsin may be reabsorbed into the blood stream after its secretion by the peptic cells into the lumen of the stomach. On the other hand, the enzyme may be absorbed into the blood directly from the secreting cells themselves without a preliminary secretion into the lumen. To test these hypotheses, gastric pepsin was fed to healthy adults, to patients with no uropepsin excretion (pernicious anemia) and to normal dogs (Figure 3).

When pepsin was fed to normal subjects already excreting appreciable amounts of uropepsin, no increases were noted in their uropepsin excretions beyond the limit of those fluctuations normally observed from day to day. On repeated occasions several patients with pernicious anemia ingested 5 grams of a potent preparation of pepsin. If as little as 0.1% of the amount of pepsin ingested eventually had been excreted into the urine, a very large quantity of uropepsin should have been excreted. Although the urine of such patients was collected for several days after the ingestion of the enzyme, at no time was it possible to detect an excretion of uropepsin by such individuals. Similarly, the administration of pepsin *per os* to dogs who normally excrete low amounts of uropepsin failed to result in an increase in these values.

In order to investigate the fate of pepsin after its introduction into the blood stream, dogs were

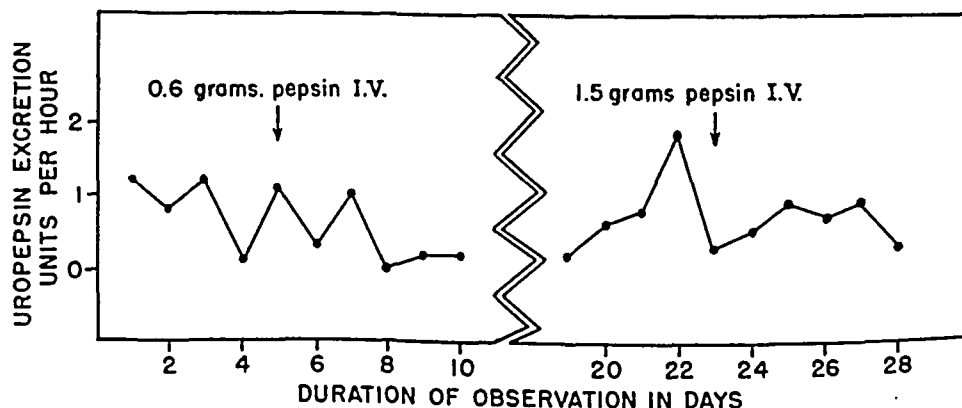


FIG. 4. EFFECT OF INTRAVENOUS INJECTION OF PEPSIN ON UROPEPSIN EXCRETION OF DOG

1:22,000 Wilson pepsin injected intravenously on days indicated by arrows after end of periods of collection of urines on respective days. Results obtained from analysis of entire 24-hour urine output.

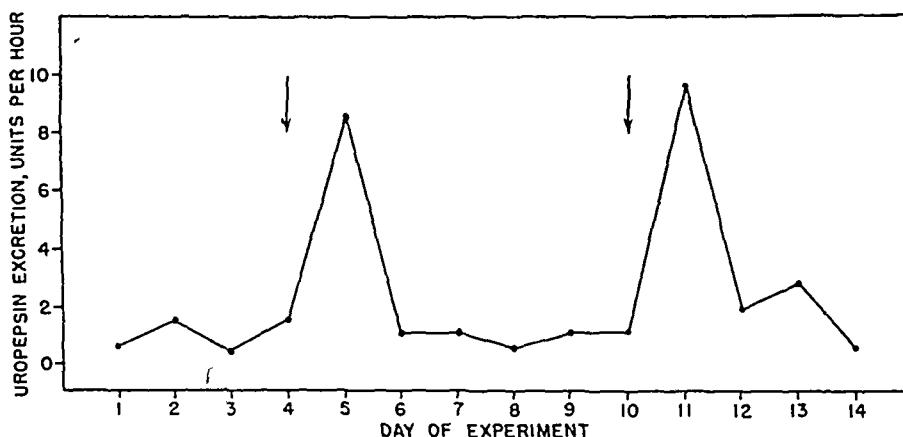


FIG. 5. EFFECT OF INTRAVENOUS INJECTION OF PEPSINOGEN ON UROPEPSIN EXCRETION OF DOG

Results obtained from analysis of entire 24-hour urine output each day. On days indicated by arrows, a crude pepsinogen solution, prepared as described in text, was injected intravenously at end of period of collection of urine for indicated day. Increases in excretion therefore occurred during a 24-hour interval following injection.

injected intravenously with as much as 80 mg. of pepsin per kg. of body weight and their urine was collected for several days thereafter. Assay of such urines failed to demonstrate an increase in uropepsin excretion (Figure 4). In view of such results, it would appear that pepsin either is not absorbed from the lumen of the stomach into the blood stream, or if so, it is inactivated in the circulating blood and is excreted by the kidney in an inactive form.

Since the preceding results tend to indicate that pepsin is not responsible for the excretion of uropepsin, it became pertinent to investigate the possibility that pepsinogen might act as the precursor of uropepsin. Accordingly, a crude specimen of pepsinogen was prepared from swine gastric mucosa according to the methods outlined by Herriot (6). A quantity of this preparation was injected intravenously into a dog. Since the dog, as demonstrated above, fails to excrete large quantities of uropepsin and since it was demonstrated that intravascular pepsin is not excreted, the intravenous injection of pepsinogen into such an animal was considered as an ideal test for the solution of this problem.

The results obtained from this experiment are illustrated in Figure 5 which demonstrates the marked increase of uropepsin excretion after the intravenous injection of pepsinogen. Such results confirm the hypothesis that pepsinogen can be excreted in the urine if it once gains entrance into the blood stream.

DISCUSSION

Our data confirm earlier studies to the effect that an enzyme with peptic activity is normally present in human urine. Most, if not all, of the proteolytic activity in urine manifest at both pH 1.5 and pH 3.5 can be attributed to the activity of a pepsin-like enzyme.

The experiments described herein suggest the source and nature of uropepsin. Lack of uropepsin activity in the urine of gastrectomized dogs (4), gastrectomized cats (2) and gastrectomized man, conclusively demonstrates that uropepsin is not found in the absence of the stomach. Furthermore, in the presence of an achylia gastrica, no uropepsin is excreted in the urine (3). It therefore may be concluded that uropepsin undoubtedly originates as the result of the peptic activity of the gastric mucosa. The possibility that such activity excites or activates the formation and secretion of uropepsin by another organ cannot be ruled out. Such an hypothesis, however, is not supported by sufficient evidence to warrant further consideration. Consequently, it is probably safe to assume that uropepsin is derived directly as the result of the function of the peptic glands in the gastric mucosa.

The peptic glands of the stomach secrete a pro-enzyme, pepsinogen, which undergoes autocatalytic conversion to active pepsin in the presence of the hydrochloric acid present in the lumen of the stomach (6). Since the normal organ contains considerable acid, most of the enzyme secreted into

the stomach must be converted almost immediately to the form of pepsin. In the normal stomach, therefore, there is little opportunity for pepsinogen to exist for a period of time long enough to permit the absorption of appreciable amounts into the blood stream. This hypothesis receives support from our own observations that the oral administration of pepsinogen with or without a buffer failed to increase uropepsin excretion in the dog. Such results can be interpreted only by assuming either that pepsinogen cannot be absorbed from within the lumen of the stomach or else that it is converted too rapidly to pepsin which latter, as was already demonstrated, cannot act as a precursor of uropepsin. Furthermore, since pepsin is not excreted as uropepsin even after its intravenous administration, it seems certain that uropepsin cannot be derived from a peptic enzyme, once the latter has been secreted into the lumen of the stomach.

The above considerations induce one to regard favorably the hypothesis that uropepsin originates as a result of the secretion of an enzyme system from the peptic glands directly into the blood (2). Inasmuch as pepsin per se does not exist in the secreting glands, it appears probable that the immediate precursor of uropepsin must be pepsinogen which is absorbed directly into the blood stream from the secreting cells and so transported intravascularly to the kidney. Since the intravenous administration of pepsinogen does result in an increase in uropepsin excretion, a fact which indicates the ability of the kidney to excrete pepsinogen, this observation lends additional support to the possible identity of uropepsin and pepsinogen.

If the above hypothesis is true, uropepsin may be regarded in the light of an excretion product of an endocrine rather than an exocrine secretion since, in this case, the product of glandular activity is being secreted directly into the blood stream rather than into the lumen of a gland. The endocrine activity of the peptic glands may be regarded somewhat analogous to a similar activity of the pancreatic acinar tissue. Although it is true that such cells secrete most of their enzyme products into the pancreatic ducts, some of these products (amylase, lipase) gain access directly into the blood and, in certain conditions, the amounts so absorbed become markedly increased.

SUMMARY AND CONCLUSIONS

1. A simple, reliable method has been described for the estimation of uropepsin excretion by man.
2. The stability of uropepsin has been confirmed and the fact that urine contains no inhibitors of uropepsin has also been demonstrated.
3. The proteolytic activity of urine at acid reactions can probably be ascribed primarily to the activity of uropepsin.
4. The presence of gastric secretory activity is essential for the formation of uropepsin.
5. Evidence is presented to indicate that uropepsin is derived from the direct secretion of pepsinogen into the blood stream by the secreting glands themselves and not from the reabsorption of pepsin from the lumen of the stomach.

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UROPEPSIN EXCRETION BY MAN. II. UROPEPSIN EXCRETION BY HEALTHY MEN^{1,2}

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In another communication (1) we presented evidence that the excretion of uropepsin in the urine is dependent upon the secretion of pepsinogen directly into the circulation by the peptic cells of the gastric mucosa. Consequently, it was postulated that the amount of uropepsin appearing in the urine might be related to the rate of "intrinsic" or endocrine activity of the peptic cells of the gastric mucosa. The present communication deals with our attempt to define those factors which regulate uropepsin excretion by healthy men.

METHODS

Twenty-seven apparently healthy men were utilized as subjects for this investigation. Urine samples were collected daily from some of these men throughout the entire 24-hour period of the day for as long as three months without interruption. In many cases, the urine formed during the hours of activity was collected separately from that formed during the hours of sleep and separate uropepsin determinations were performed on the individual samples. In other subjects, only the urine formed during the hours of sleep was collected and assayed. Notations of these differences in procedure appear in the appropriate places in the text and protocols. The methods employed for the collection of urine and the determinations of uropepsin activity have been described in the preceding paper (1). Throughout this and subsequent reports of this series, the unit of uropepsin activity will be defined as that amount which, in the standard 10-minute assay, is required to release 1 mg. of acid-soluble tyrosine-like material.

In order to express the uropepsin excretion in terms of units per hour, the total amount of uropepsin excreted was divided by the number of hours during which the sample was collected. The presentation of uropepsin excretion in rates rather than amounts per sample provides a basis for the comparison of uropepsin excretion using specimens collected over varying periods of time.

Since the collection of specimens of urine formed only during the sleeping hours presents the minimum incon-

venience to subjects, the routine collection of such specimens could be obtained for a number of consecutive nights from a large group of men. As will be demonstrated subsequently, such samples may be used accurately to determine the uropepsin excretion for the entire 24-hour period.

RESULTS

Expression of data

The amount of uropepsin excreted per hour by any particular subject was found to vary from day to day. Although such fluctuations in excretion in any one subject were relatively minor in character, it became essential to determine whether or not the different rates of uropepsin excretion from a subject could furnish a reliable estimate of his true excretion pattern. This can be determined by the application of conventional methods of statistical analysis. Since such methods are most efficient when applied to data that can be arranged in the form of a normal distribution frequency curve, it became essential to determine first whether or not the uropepsin excretion values of each subject were so distributed. Inasmuch as the data obtained from the analysis of night specimens of urine will form the basis for a great majority of our conclusions, such data were utilized to investigate the distribution frequency of the uropepsin excretion values in any one subject.

When the cumulative percentage frequency of a variable is plotted on probability paper against the units of that variable, the plot is a straight line when the distribution is normal. A convenient method of making the comparison is to transform the percentages to probability units, or probits (2). Typical results of the probit plots are illustrated graphically in Figure 1 which demonstrates the distribution frequency in the case of three separate subjects. When the data representing the arithmetic units of uropepsin excretion per hour were plotted against the probits, a straight line was not obtained, *i.e.*, the excretion values for uropepsin,

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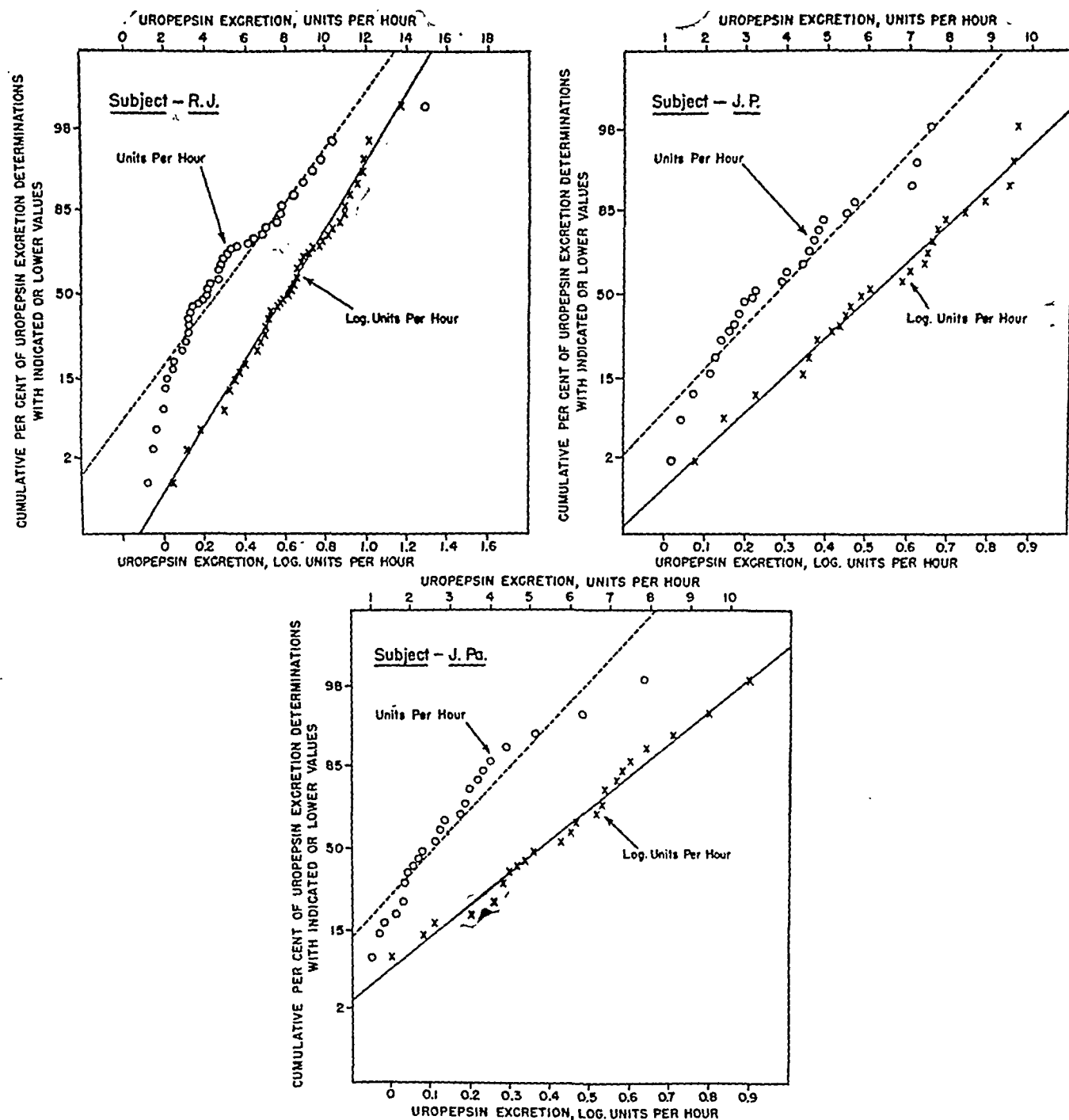


FIG. 1. FREQUENCY DISTRIBUTIONS OF UROPEPSIN EXCRETION VALUES

Probit plots of uropepsin excretions of three subjects in terms of units per hour and logarithms of units per hour. Each point represents an excretion value obtained from the analysis of a "night" specimen of urine. The straight lines have been fitted to the respective data by inspection and indicate that the systematic deviations are removed by the logarithmic transformations.

expressed as units per hour, did not group themselves in the form of a normal distribution curve. However, when the same procedure was repeated but the excretion results expressed exponentially in terms of the Briggsian logarithms of the units of uropepsin per hour, the various data were fitted well by a straight line without the systematic deviation that was uniformly characteristic of the

arithmetic plots (Figure 1). Straight lines were obtained from the probit plots of the logarithmic uropepsin excretion values for each of the other subjects. It is worth noting that the use of the values obtained from the analysis of the entire 24-hour urine collection samples yielded similar results to those obtained from night specimens.

The preceding analysis shows that in order to

compare efficiently the uropepsin excretion patterns of different subjects or the excretion values in the same subject from one time to another, the excretion of uropepsin should be expressed in terms of the logarithms of the units (log units), rather than in terms of the arithmetic values. Therefore, all uropepsin excretion values in this and succeeding reports will be expressed in the terms of the logarithms of the units.

Influence of urine volume

Inasmuch as the rate of urine formation often varied markedly during the period of collection of the specimens, it appeared pertinent to determine if the fluctuations observed in uropepsin excretion were due to differences in the rate of urine formation. Toward this end, the results of consecutive uropepsin assays on 13 different subjects were

examined in order to determine whether or not uropepsin excretion was a function of the volume of samples of urine. Conventional methods (3) were utilized to determine the linear regression equations expressing the best fit of the data concerning the relation of log units excreted to the volumes of the urine samples.

Figure 2 represents the regression curve corresponding to the experimental observations drawn from assays performed on samples collected for 66 consecutive nights on one subject. Such results are typical of those obtained from all other subjects and no differences were noted, irrespective of whether the data were obtained from the analysis of 24-hour specimens or from analysis of samples representing urine formed during either sleeping or waking hours. Visual inspection indicates the lack of dependence of uropepsin excretion on the rate of urine formation. The calculated regression coefficients for all 13 subjects are recorded in Table I together with the statistics used to test the significance of those coefficients.

Although it is conceivable that uropepsin excretion might be in part a function of the rate of urine formation, the regression may not account for an appreciable portion of the total variation in daily uropepsin excretions even though it is statistically significant. The percentage of the total

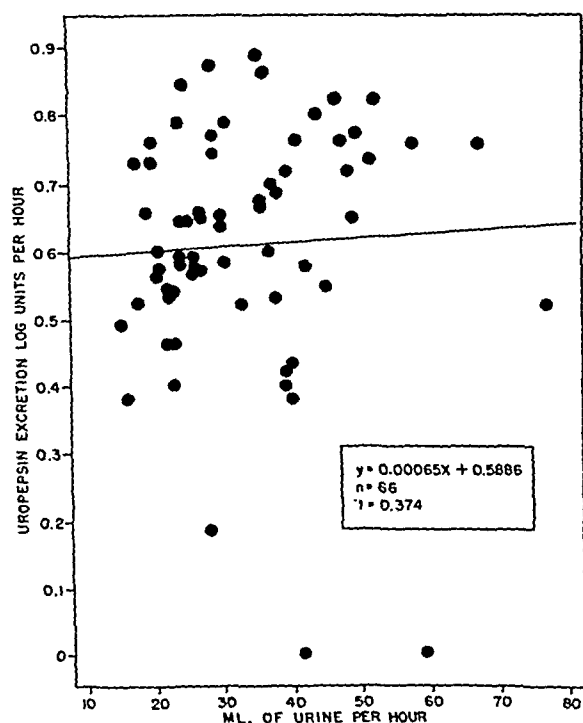


FIG. 2. RELATION OF URINE VOLUME AND UROPEPSIN EXCRETION

Each point represents results obtained from a single specimen of "night" urine. The equation defines the straight line that most closely expresses the relationship between volume of urine and rate of uropepsin excretion for all samples. The "t" test indicates the lack of significance of this relationship. The number of night specimens is given by the figure corresponding to "n."

TABLE I

The relation between uropepsin excretion and urine volume

Subject	No. of assays	Regression coefficient	"t"	V_1/V_2 per cent
I. W.	31	0.00066	0.537	1.0
L. R.	66	0.00065	0.374	1.3
V. G.	15	0.00212	1.191	6.1
W. M.	16	0.01003	3.039*	48.4
J. A.	11	-0.00196	0.797	3.6
H. K.	12	0.00674	3.179*	89.9
J. P.	28	0.00030	0.320	0
J. W.	29	0.00397	1.449	2.8
A. F.	190	0.00046	0.694	2.0
I. H.	26	-0.01228	1.971	11.2
E. J.	26	-0.00055	0.420	3.3
A. S.	15	0.01004	1.764	9.0
E. S.	14	0.00560	1.618	12.4

The uropepsin excretion is expressed as log units per hour. The linear regression coefficient indicates the calculated increase in the rate of uropepsin excretion anticipated from each ml. increase in urine volume. * = Regression coefficient is significant at 5% level; otherwise "t" values indicate no significance of regression coefficients. The ratio between the variance due to the regression and the total variance is listed as V_1/V_2 and is expressed as the percentage of the total variance accounted for by the regression.

variance in uropepsin excretion for each individual that could be accounted for by the regression of the log rate on the volume was calculated. A summary of the results of such calculations appears in Table I. The "t" tests (3) indicate that in only two subjects was uropepsin excretion found to regress to a significant degree on urine volume. Likewise, in only the same two subjects could the calculated, theoretical regression between volume and uropepsin excretion explain an appreciable percentage of the total variance in all of the excretions in any one subject. This fact is illustrated by the statistics in the column " V_1/V_2 " which indicate the percentage of the total variance attributable to the theoretical regression (3). These results indicate that, in the overwhelming majority of subjects, variations in urine volume play no significant role in the regulation of uropepsin excretion. Furthermore, in those two subjects in whom it was found to play an apparent role, only a relatively small number of assays had been performed. Consideration of all of these factors leads to the conclusion that for all practical purposes, variations in urine volume can be neglected, and that the formulation of results in terms of rate of excretion furnishes a better index of the various factors regulating uropepsin excretion than does their expression in terms of the concentration of the enzyme as it is found in the urine.

Similarly, analysis of the results has indicated the essential independence of uropepsin excretion on factors such as the pH or the specific gravity of the urine.

Constancy of uropepsin excretion

The expression of uropepsin excretion in terms of the rate forms the basis for an efficient method for the analysis of samples collected over varying fractions of the same or different days with the provision that uropepsin is excreted at a relatively constant rate throughout the 24-hour period.

The constancy of the rate of excretion of uropepsin was investigated by two procedures. In the first of these, a comparison was made between uropepsin excretion by the same subject during the night and during the day. For this purpose, the urine formed by individual subjects during the waking hours ("day urine") was collected and assayed separately from that formed during the hours of sleep ("night urine") for a number of consecutive 24-hour periods. The significance of the differences between the log units of uropepsin excreted per hour in the night and day urines of five subjects chosen at random was estimated by calculation of the "t" values corresponding to the differences between the means of the uropepsin excretion for all of the waking and all of the sleeping hour specimens. These results and their interpretations are found in Table II.

The number of consecutive 24-hour periods during which urine was collected in separate day and night fractions for the above-mentioned subjects averaged 31 days with extremes of nine and 53 days. In none of these subjects was the difference between the mean day and night excretions found to be significant at the 5% level. Indeed, the dif-

TABLE II
Comparison of uropepsin excretion during waking and sleeping hours

Subject	No. of days	Uropepsin excretion (log units per hour)				Difference of means	“t”	“Probabilities”†		Significance of Differences
		Waking hours		Sleeping hours				5%	1%	
		Mean	S.D.*	Mean	S.D.*					
A. M.	9	0.35	0.24	0.37	0.20	0.02	0.185	2.12	2.92	none
H. W.	21	0.64	0.14	0.64	0.14	0	0	2.02	2.70	none
L. R.	53	0.66	0.39	0.60	0.44	0.06	0.53	1.98	2.63	none
J. F.	36	0.87	0.49	0.74	0.41	0.13	0.91	1.99	2.65	none
S. B.	37	1.01	0.13	0.94	0.15	0.07	1.538	1.99	2.65	none

Uropepsin excretion values obtained from separate analyses of specimens of urine formed during waking and sleeping hours of the same 24-hour period.

$$* \text{S.D.} = \pm \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

† The probabilities listed are the values of "t" at which the differences between uropepsin excretion during the waking and sleeping hours become significant at the 1% and 5% levels (3).

ferences did not even approach significance except in the case of one subject. In all four other instances, the significance of the differences was found to be so low as to permit them to be completely ignored. In other words, the differences between uropepsin excretion per hour during the waking and sleeping periods were not significantly greater than the differences found to occur among excretions per hour during consecutive waking or during consecutive sleeping periods. The lack of difference between day and night uropepsin excretion was found to exist irrespective of the individual subject's characteristic rate of excretion. Thus, in these five subjects, the mean uropepsin excretion rate varied from 0.35 log units per hour to 1.01 log units per hour, a range which nearly covers the entire range of dispersion of the mean uropepsin excretion values of healthy men (Table III).

In the preceding analysis, one variable has been temporarily neglected. Since the average duration of the waking period was 16 hours and of the sleeping period only eight hours, it is conceivable theoretically that the absence of a combined effect of the variables "day" and "night," on the one hand, and of length of time of collection of urine, on the other hand, could be due to an interaction between the two factors. In other words, if the amount of uropepsin excreted were found to be some function of the duration of the period of collection of the urine sample, the influence of wakefulness or sleeping (or night or day) alone might have been concealed.

However, the constancy of the rate of uropepsin excretion and the lack of its dependence on the length of the time interval corresponding to the duration of excretion of urine in the sample was demonstrated in yet another manner. In some subjects, samples of urine, representing collections for some fraction of the 24-hour period, were obtained for a number of consecutive days. No attempt was made to advise these subjects to standardize the duration of the period of collection of the urine samples. As a consequence, the times of collection of the urine samples on consecutive days varied from as little as two hours to as much as the entire 24-hour day. When all of these data were analyzed no effect of the duration of collection of urine was revealed. Figure 3 illustrates the data obtained from one such subject and demonstrates

the lack of a significant regression. This indicates clearly that fluctuations in uropepsin excretion per hour are not influenced appreciably by the time of the day during which the urine is collected or by the duration of the period of the collection of urine.

The above results have definite practical as well as theoretical implications. The collection of 24-hour specimens of urine day after day from a number of subjects presents certain practical difficulties. On the other hand, the collection of specimens formed during the sleeping hours only is far less objectionable to the subject and constitutes an entirely feasible project. For this purpose, the subject is instructed to void and discard his urine before going to sleep. In the morning he is advised to empty his bladder completely and collect the entire specimen. The times of the two voidings are noted and it then becomes possible to determine the excretion per hour during the sleeping hours. Since such values are representative of uropepsin excretion throughout the entire 24-hour period, they constitute a valid index for the comparison of excretion between subjects and in the same subject from day to day. It was thus found possible to utilize "night specimens" of urine for the determination of each subject's uropepsin excretion pattern.

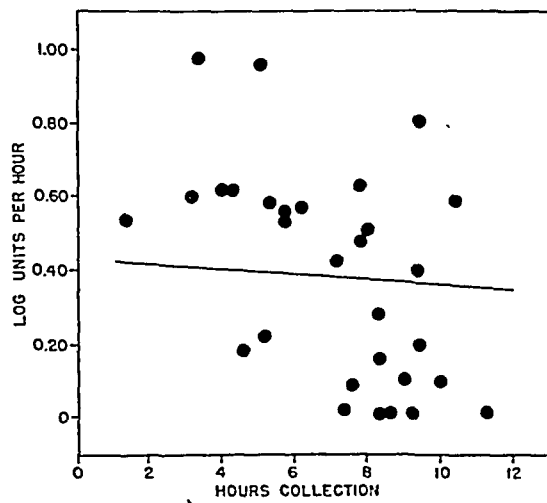


FIG. 3. EFFECT OF DURATION OF COLLECTION ON UROPEPSIN EXCRETION

Each point represents the results obtained from the assay of one of a series of consecutive night urine collections. The coefficient of linear regression is: $b = 0.0064$; $t = 0.333$.

TABLE III
Uropepsin excretion of healthy men

Subject	Age	First four assays					All assays	
		1	2	3	4	Mean	Number	Mean
S. B.	24	0.77	0.73	0.72	0.81	0.76	44	0.93
W. B.	30	0.49	0.69	0.11	0.15	0.36	5	0.41
A. D.	28	0.51	0.18	0.68	0.34	0.43	45	0.34
C. H.	27	0.59	0.52	0.08	0.97	0.54	55	0.78
M. J.	21	0.58	0.46	0.20	0.42	0.42	11	0.25
R. J.	31	0.20	0.65	0.18	0.44	0.37	127	0.62
D. J.	27	0.52	0.26	0.70	0.20	0.42	13	0.52
R. J.	31	0	0.58	0.32	0.15	0.26	5	0.21
L. L.	24	0.62	0.51	0.69	0.65	0.62	6	0.64
C. M.	29	0	0.45	0.57	0.20	0.31	49	0.35
I. A. M.	41	0.53	0.43	0	0.49	0.36	11	0.41
S. O.	24	0.36	0	0.40	0.56	0.33	47	0.36
J. P.	52	0.43	0.45	0.08	0.58	0.39	33	0.39
J. P.	24	0.60	0.64	0.23	0.65	0.53	31	0.54
L. R.	26	0.74	0.76	0.73	0.42	0.66	71	0.61
R. S.	24	0.08	0.08	0.15	0.51	0.21	4	0.21
T.	—	0.64	0	0.72	0.23	0.40	14	0.29
I. H. W.	25	0.34	0.46	0.73	0.63	0.54	83	0.65
N. W.	23	0.82	0.48	0.65	0.45	0.60	8	0.52
I. Y.	26	0.65	0.92	0.82	0.75	0.79	4	0.79
R. McC.	26	0.58	0.52	0.68	0.20	0.50	8	0.46
M. H.	24	0.38	0.42	0.65	0.62	0.52	14	0.58
B. M.	21	0.60	—	—	—	0.60	1	0.60
G. G.	50	0.07	0.71	—	—	0.39	2	0.39
K.	—	0.59	0	—	—	0.30	2	0.30
R. D.	19	0.52	—	—	—	0.52	1	0.52
H. T.	33	0.32	—	—	—	0.32	1	0.32
Mean excretion for entire group		0.46	0.45	0.46	0.47	0.46		0.48

From the preceding considerations, it is evident that the uropepsin excretion of any one subject varied from day to day but that the fluctuations were such as to cause all of the values (in terms of log units per hour) to be arranged in the form of a normal distribution curve. It then became pertinent to inquire whether or not the differences among the subjects were greater than the differ-

ences within the subjects. Furthermore, since it was not possible always to obtain specimens for the same number of consecutive periods of sleep, it became important to determine also how many consecutive collections were required to form a valid basis for the characterization of an individual's uropepsin excretion and for the comparison of excretions between different subjects. The pertinent data and conclusions are presented in Tables III and IV.

The results of 695 uropepsin excretion determinations performed on the night specimens of urine obtained from 27 healthy adult male subjects for from one to 127 consecutive nights are summarized in Table III. It is evident by inspection that irrespective of whether only the first assay, the mean of the first four assays or the mean of all of the available assays for each subject is used, the mean value of the entire group is essentially the same. Therefore, there is no sampling error and one or any number of assays on each individual can be used for making comparisons between different groups. Naturally, the usual methods for weighting for a possibly different number of assays on each individual must be used in making such comparisons, in order not to bias the estimate of group performance by giving the various individuals unequal weight.

The preceding conclusion is confirmed in more elegant form by an analysis of variance (3). The quantitative estimate of the degree of variance in uropepsin excretions within and among the various subjects is tabulated in Table IV. These statistics were derived by analysis of the data obtained from

TABLE IV
Analysis of variance of uropepsin excretion of healthy men

The variance was calculated from data obtained from the first four and the first ten consecutive assays of specimens from all subjects with the corresponding or greater number of assays.

Source of variation	Sums of squares		Degrees of freedom		Mean variance			F		
	Four samples	Ten samples	Four samples	Ten samples		Four samples	Ten samples		Four samples	Ten samples
Among all men	1.9506	3.3955	21	14	A	0.0929	0.2425	A/B	1.96*	5.91†
Within all men	3.1259	5.5317	66	135	B	0.0474	0.0410			
Among samples	0.0033	0.0212	3	9	C	0.0011	0.0024	C/D	0.02	0.05
Interaction	3.1226	5.5529	63	126	D	0.0496	0.0441			

* Significant at 5% level.

† Significant at 0.1% level.

the results of the first four consecutive night urine specimens of all subjects from whom four or more such specimens were obtained, and from the first ten consecutive night urine specimens of all subjects from whom ten or more specimens were obtained. In that manner, the bias that comes from unequal weighting is avoided.

It is apparent that the mean variance within the subjects in the rate of uropepsin excretion (0.0474) is smaller than the mean variance in uropepsin excretion among all the subjects (0.0929). The ratio of these two variances ($F = 1.96$) is greater than would be expected on the basis of chance, 95 times out of 100. In other words, each man has a uniform pattern of excretion that is characteristic for him. Also, the differences in the mean uropepsin excretion of different subjects far exceed those that could be attributable to the fluctuations in uropepsin excretion that occur from day to day in the group, as well as in each man. This conclusion is derived from the lack of a significant ratio ($F = 0.02$) of the variance among samples to the variance due to "interaction." The "interaction" is the mean variation remaining after the principal sources of variation (among and within men, and among samples) have been removed, and is due to uncontrolled sources which are assumed to act at random. Thus, the analysis shows that men differ significantly from each other in the rate at which they excrete uropepsin and that there is no first or subsequent sample effect.

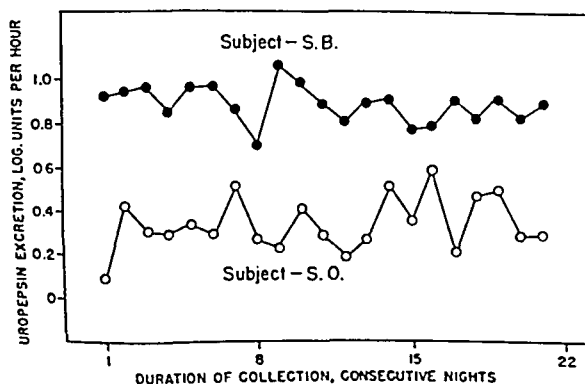


FIG. 4. UROPEPSIN EXCRETION PATTERNS OF TWO SUBJECTS

Each point represents a value obtained from analysis of a "night" specimen of urine. The figure illustrates results obtained from such urines collected for 21 consecutive nights.

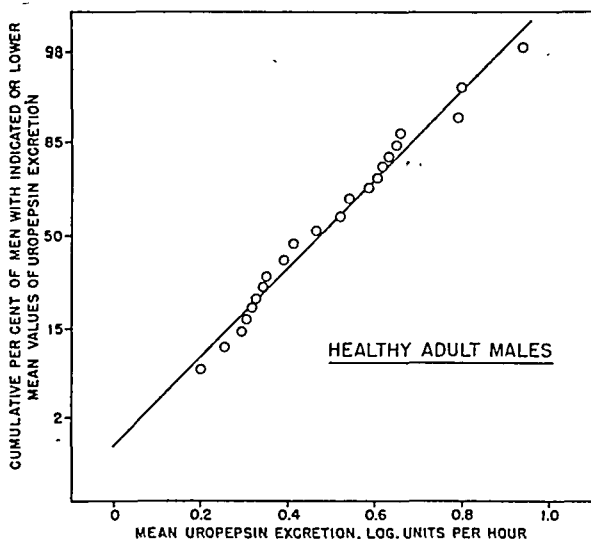


FIG. 5. FREQUENCY DISTRIBUTION OF MEAN UROPEPSIN EXCRETIONS OF HEALTHY MEN

Probit plots of the mean uropepsin excretion values of all assays for each subject. The straight line corresponding to these points has been fitted by inspection.

The data obtained from the first ten night urine specimens of all subjects with ten or more consecutive night specimens reveal essentially the same mean variance within each subject but a greater variance among the subjects, so that the ratio of the variance among subjects to that within subjects becomes even more significant. Thus it is evident from the analysis of both four and ten samples that there is a relatively small variance in uropepsin excretions per hour within each subject and therefore that the daily uropepsin excretion pattern appears to be characteristic of the individual. This fact is illustrated graphically in Figure 4 in which two uropepsin excretion rates during consecutive nights are compared. For this example, two subjects were chosen, one of whom excreted uropepsin at a high rate, the other of whom excreted uropepsin at an appreciably lower rate.

In order to investigate the nature of the observed variance among subjects, the probit transformation was utilized; the means of all of the available assays of the night urines of each subject, in terms of log units per hour, were plotted against their cumulative frequencies (Figure 5). The lack of systematic deviation and the relatively small scatter makes it evident that the population is distributed normally. Since the previous analyses indicate

that the group of subjects chosen is representative of the entire population of healthy adult males and constitutes a random sample chosen without bias, the distribution of their mean uropepsin excretions in the form of a normal curve indicates that the subjects are a homogeneous group with a mean rate of uropepsin excretion of 0.48 log units per hour.

DISCUSSION

A number of observations indicate that uropepsin is excreted at a fairly constant rate by the healthy adult male. The constancy of the rate of excretion throughout the same 24-hour period is indicated by the lack of a significant difference between excretion rates during the waking and sleeping hours (Table II) and by the fact that the excretion rate is not appreciably affected by wide differences in the duration of the collection period of the urine (Figure 3). This fact justifies the determination of the uropepsin excretion rate from analysis of a sample of urine representing the urine excretion of but a fraction of the entire day and also validates the comparison of excretion rates as determined from samples of urine obtained during different lengths of periods of collection and at a different time of the day. The constancy of the rate of excretion of any one subject from day to day was demonstrated by the analysis of the data summarized in Tables III and IV and presented graphically in Figure 4.

The small variance within subjects is extremely significant in spite of the fairly wide fluctuations in daily excretion rates that may be noted in some instances. It indicates the reliance that may be placed on the results obtained from analysis of a single specimen of urine. Thus, if the expected uropepsin excretion rate was calculated from the assay of only one specimen from a healthy man, the result would, on the average, have a relative standard deviation of ± 62 per cent. That is, it would closely approximate his true uropepsin excretion rate. This figure may be compared to the relative standard deviation of the differences among the men of ± 58 per cent.

The degree of constancy demonstrates that the rates are grouped in a characteristic excretion pattern. However, failure to utilize the true extent of random fluctuation may easily lead to false conclusions such as that "night" excretions may differ from "day" excretions. Furthermore, the degree

of constancy of the rate of uropepsin excretion may be concealed by expressing it in terms of units per day or per hour if the logarithmic nature of the distribution is neglected. Comparisons using statistics based on the assumption that the rates are distributed normally arithmetically are not efficient and, furthermore, expression of values as log units "smooths out" apparent differences.

It is pertinent to inquire concerning the causes of this constancy of uropepsin excretion. At the onset of our study, we anticipated that marked differences might be found between day and night excretions as was suggested by Gottlieb's data (4). Bucher also conceded the possibility that higher than usual rates in the daily uropepsin excretion of some of her subjects could be attributed to increased wakefulness coincident with the pressure of classroom examination (5). However, examination of our data (Table II) reveals no significant diurnal variation. Furthermore, regardless of variations in the number of hours during successive days, during which our subjects were awake or asleep, each subject exhibited a fairly constant rate of excretion. Thus, it is evident that sleep and wakefulness per se exert very little influence on uropepsin excretion. Furthermore, in spite of variations, from day to day, in the amount and type of work, exercise or recreation, the constancy of the rate of uropepsin excretion is indicative of the lack of influence of these latter factors on the regulation of uropepsin excretion.

The lack of diurnal fluctuations also suggests that the influence of the usual meal, varying as it does from day to day in quality and quantity, on uropepsin excretion must be minimal for otherwise, if eating had caused either an increase or decrease in the uropepsin excretion rate, the day and night urine values would have been different. It is conceivable that if no food had been ingested during the daytime, the uropepsin excretion rates would have been lower than those found at night. In an attempt to examine more closely the effect of eating, fractional urine specimens have been collected from a few subjects throughout the waking hours and assayed for uropepsin. These experiments failed to reveal any significant fluctuations that could be attributed to the ingestion of food.

However, the influence of food on the secretion of gastric juice is well-known. Since our data indicate that the ingestion of food does not play any significant role in the regulation of uropepsin ex-

cretion, it becomes evident that a dissociation may exist between the stimulation of the secretion of gastric juice into the stomach and the excretion of uropepsin. Such a conclusion is in accord with the observation that caffeine and histamine, both of which are powerful stimulants of gastric secretion, exert no influence on the excretion of uropepsin by the cat (6).

The apparent dissociation between gastric secretion and uropepsin excretion may reflect a dissociation between the rates of pepsinogen secretion into the circulation and pepsinogen secretion into the lumen of the stomach. Consequently, such factors as do influence uropepsin excretion may do so by affecting the endocrine-like function of the peptic glands of the stomach rather than the exocrine functions of these glands.

Another factor that appears to exert very little influence on uropepsin excretion is the rate at which urine is formed by the apparently healthy kidney. The data in Table I and Figure 2 indicate that the amount of uropepsin excreted is independent of marked variations in the volume of urine excreted. This conclusion is supported also by the lack of diurnal fluctuation in uropepsin excretion (Table II) in spite of the fact that night urine generally is more concentrated and lower in volume per unit of time than is urine excreted during waking hours. Furthermore, very little fluctuation has been observed in uropepsin excretions by the same subject from winter to summer even in the presence of marked changes in urine concentrations.

All of these findings substantiate the conclusion of Bucher (5), that the total amount excreted per unit of time, rather than the concentration, characterizes uropepsin excretion by the human subject. However, all of our data, particularly that presented in Table II, fail to support Bucher's contention that a single specimen of urine cannot be used with validity for the determination of the rate of uropepsin excretion. Our own observations support the conclusion that, if the duration of the time of collection of the specimen is known, the rate of uropepsin excretion can be calculated accurately for the entire day from the analysis of a single specimen containing the urine formed during some fraction of the entire 24-hour period.

As yet, it has not been possible to ascribe to any single factor the responsibility for the differences in excretion rates exhibited by different people.

However, the very fact that all of these subjects comprise a homogeneous group in regard to their uropepsin excretion (Figure 5) would tend to indicate that the same group of factors operate to regulate the uropepsin excretion of all the subjects.

SUMMARY AND CONCLUSION

1. Uropepsin excretion by healthy men is best expressed in terms of rates of excretion. Comparisons among rates may be made efficiently by using the logarithms of the rates.

2. Healthy men excrete appreciable quantities of uropepsin at a fairly constant rate throughout the day and from day to day. This rate is not markedly affected by the volume, specific gravity or acidity of the urine or by factors such as wakefulness, sleeping, or ordinary exercise and the usual fluctuations in dietary habits. The ingestion of food does not appear to stimulate uropepsin excretion.

3. The rate of uropepsin excretion is characteristic of the individual subject.

4. Healthy men form a homogeneous group in regard to their rates of uropepsin excretion.

5. Evidence is presented that uropepsin excretion is a function of the endocrine rather than the exocrine activity of the peptic glands of the stomach.

ACKNOWLEDGMENT

We wish to acknowledge the critical assistance of Dr. Henry W. Ryder of the Kettering Laboratory of Applied Physiology who suggested the statistical methods used in the analysis of the data, and the aid rendered by Dr. I. H. Weiland.

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UROPEPSIN EXCRETION BY MAN. III. UROPEPSIN EXCRETION BY PATIENTS WITH PEPTIC ULCER AND OTHER LESIONS OF THE STOMACH^{1, 2}

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In preceding papers (1, 2) we have outlined results which indicate that uropepsin excretion may serve as a useful index of the rate of the "intrinsic" or endocrine-like activity of the pepsinogen-secreting cells of the gastric mucosa. Data were also presented concerning the uropepsin excretion patterns of healthy, adult male subjects. Since various lesions of the stomach and duodenum are associated with either an increase or a decrease in the rate of gastric activity, the uropepsin excretions of patients suffering from a variety of conditions affecting the gastrointestinal tract were investigated.

METHODS

The urine was collected and assayed according to the procedure described in a previous communication (1). The unit of uropepsin used is the same as defined earlier, namely, that amount of uropepsin which, in the standard ten-minute assay, liberates 1 mg. of "tyrosine-like" acid soluble material.

The patients utilized in the study included only those whose diagnosis had been established by means of objective criteria universally accepted as adequate for this purpose. Inasmuch as the opportunity has not yet been presented to study a sufficiently large number of healthy women and since our previous study of healthy subjects was confined to men, this report will be limited except where otherwise noted, to the presentation of results obtained from male patients only.

RESULTS

In the preceding paper (2), some of the factors that define and characterize the uropepsin excretion of healthy men were discussed. The influence of these factors was revealed by the application of statistical procedures to the assembled data. The

data reported herein were subjected to the same types of analysis as have been utilized in the preceding paper. The results of these statistical analyses revealed the fact that certain of the conclusions obtained from the study of uropepsin excretion of healthy men can be extended to the results obtained from the patients considered in this paper. Thus, as with the group of normal men, so with the present group of patients it was possible to demonstrate that (a) data concerning uropepsin excretions should be expressed as log units per hour rather than in terms of the arithmetic values; (b) data concerning the logs of the units of uropepsin excretions of any one patient, from day to day, group themselves around a mean value in the form of a normal distribution curve; (c) uropepsin excretion is not markedly affected by wide fluctuations in the rate of urine formation; (d) diurnal fluctuations in uropepsin excretion fall well within the limits of the fluctuations observed from day to day and, accordingly, uropepsin excretion remains relatively constant throughout the day.

In consideration of these facts, uropepsin excretions will be expressed only in terms of the log units per hour. In view of the lack of an essential difference between uropepsin excretion in urine formed during the waking hours and during sleeping hours of the same day, and in view of the marked advantages inherent in the collection and analysis of specimens formed only during the hours of sleep (2), we shall present data obtained solely from the analysis of "night urines." However, it should be noted that similar results were obtained from the collection of "day urines," in those patients in whom the collection of such samples was found to be a practical procedure.

A. Peptic ulcer

Uropepsin assays were performed on 695 "night" specimens of urine obtained from 30 pa-

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tients with benign gastric or duodenal ulcers. In every one of these subjects the diagnosis had been established as the result of unequivocal roentgenologic, gastroscopic or post-operative histopathologic examination of the lesion. The results of all of the uropepsin determinations performed on night specimens of urine obtained from these 30 male patients for from one to 207 consecutive nights are summarized in Table I and reveal that the mean excretion is approximately twice that observed in healthy men (2).

As was found to be the case with normal subjects, no sampling error has been introduced as far as the entire group of ulcer patients is concerned. The mean value for the uropepsin excretion per hour for the entire group was not found to be radically changed if only the first assay, the mean of the first four assays, or the mean of all of the available assays for each subject is used for the calculation of the mean uropepsin ex-

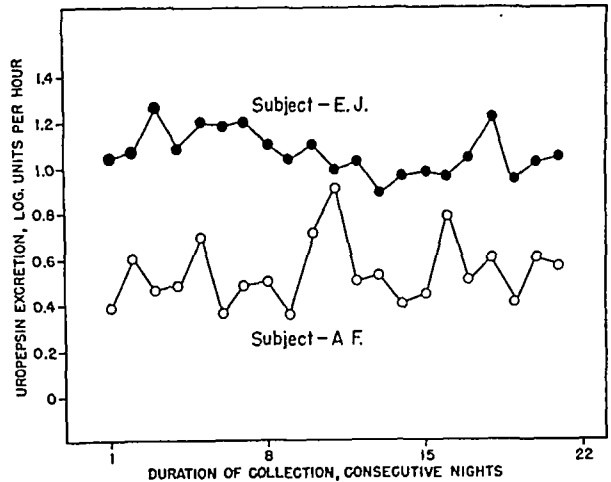


FIG. 1. UROPEPSIN EXCRETION PATTERNS OF TWO ULCER PATIENTS

Each point represents a value obtained from analysis of a "night" specimen of urine. The illustrated results were obtained from such urines collected for 21 consecutive nights.

cretion for the entire group. It is apparent, therefore, that a single sample taken from each of a group of men may be used to determine whether a group of such individuals excrete uropepsin at a rate similar to that characteristic of the group of male patients with peptic ulcers.

In order to derive a quantitative estimate of the significance of the observed fluctuations in uropepsin excretions within and among subjects, an analysis of variance (3) was performed on the data summarized in Table I. For this purpose, variances were calculated on the basis of the first four assays of all ulcer patients with four or more assays and on the basis of the first ten assays of all patients with ten or more assays. The results of these calculations are presented in Table II. For the sake of comparison of groups, similar calculations on data from healthy males, taken from Table IV of the preceding paper (2), have been included.

As was anticipated, the results of both sets of calculations essentially confirmed one another; the significance of certain differences became more apparent in the analysis using the greater number (ten) of samples. It is obvious that the variance within men with ulcer was much less than the variance among men with ulcer and the chances are less than one in a hundred and one in a thousand that this could be due to random sampling. Such

TABLE I

Uropepsin excretion of male patients with peptic ulcer

Subject	First four assays					All assays	
	1	2	3	4	Mean	Number	Mean
J. C.	.76	1.03	.40	1.03	.81	8	.96
A. F.	.38	.70	.62	.88	.65	207	.63
J. H.	.53	.56	.63	.69	.60	46	.66
E. J.	1.06	1.03	1.03	1.03	1.04	64	.96
A. P.	.60	.60	.52	.51	.56	13	.54
L. R.	.72	.68	.96	.76	.78	14	.88
J. S.	.49	.91	.56	.80	.69	13	.65
Si.	.26	.88	.81	.56	.63	42	.68
P. S.	.28	.30	.11	.92	.40	16	.62
J. A.	.28	.67	1.07	1.28	.83	9	.70
J. A.	1.05	1.55	.76	1.31	1.17	4	1.17
P. C.	.81	.61	—	—	.71	2	.71
H. C.	.90	—	—	—	.90	1	.90
T. C.	1.14	.98	1.05	.99	1.04	7	.99
Da.	.87	.30	—	—	.59	2	.30
J. D.	.83	.74	.99	.83	.85	5	.82
D. E.	.40	.59	.56	.54	.52	5	.56
S. F.	1.43	.93	.58	.60	.89	6	.78
V. G.	.91	.75	.95	1.06	.92	10	.88
H. G.	.57	.95	.56	—	.69	3	.69
G. K.	1.06	.79	—	—	.93	2	.93
H. K.	.53	.86	.76	.57	.68	12	.49
W. M.	1.02	.58	.88	.89	.84	16	.96
L. M.	.76	—	—	—	.76	1	.76
J. R.	1.17	—	—	—	1.17	1	1.17
J. S.	.46	.45	.90	.32	.53	6	.58
J. W.	.58	.68	.82	.75	.71	29	.67
Zi.	1.32	.45	.86	1.24	.90	4	.90
Ne.	.76	—	—	—	.76	1	.76
M. G.	.30	.20	.95	.93	.60	10	.63
Mean excretion	.74	.72	.75	.84	.76		.76

TABLE II

Analysis of variance in uropepsin excretion of men with peptic ulcers

The variance was calculated from data obtained from the first four and first ten consecutive assays of specimens from all subjects with the corresponding or greater number of assays. The statistics for the healthy men were taken from Table IV of the preceding paper (2). "All men" refers to the sum of ulcer patients plus healthy men.

Source of variation	Sum of squares		Degrees of freedom		Variance			F		
	Four samples	Ten samples	Four samples	Ten samples		Four samples	Ten samples		Four samples	Ten samples
Among all men	8.8605	10.4891	43	27	A	0.2061	0.3885	A/B	3.77†	8.65†
Within all men	7.2127	11.3219	132	252	B	0.0546	0.0449			
Among samples	0.1211	0.2620	3	9	C	0.0404	0.0291	C/D	0.73	0.64
Interaction	7.0916	11.0599	129	243	D	0.0550	0.0453			
Ulcer patients—healthy men	3.7127	4.2697	1	1	E	3.7127	4.2697	E/B	68.00†	95.09†
Among ulcer patients	3.1971	2.8239	21	12	F	0.1522	0.2353	F/G	2.46†	4.75†
Within ulcer patients	4.0869	5.7901	66	117	G	0.0619	0.0495			
Among samples	0.2442	0.3744	3	9	H	0.0814	0.0416	H/I	1.33	0.83
Interaction	3.8427	5.4157	63	108	I	0.0610	0.0501			
Among healthy men	1.9506	3.3955	21	14	J	0.0929	0.2425	J/K	1.96*	5.91†
Within healthy men	3.1259	5.5317	66	135	K	0.0474	0.0410			
Among samples	0.0033	0.0212	3	9	L	0.0011	0.0024	L/M	0.02	0.05
Interaction	3.1226	5.5529	63	126	M	0.0496	0.0441			
Total	16.0732	21.8110	175	279		0.0918	0.0782			

* Significant at 5% level. † Significant at 1% level. ‡ Significant at 0.1% level.

findings indicate that each ulcer patient excreted uropepsin at a fairly characteristic rate subject, of course, to minor fluctuations from day to day. The differences among excretion rates of peptic ulcer patients are illustrated graphically in Figure 1 which compares the uropepsin excretion rates per hour of two particular patients during consecutive nights.

The distribution of the mean uropepsin excretion rates of the ulcer patients is graphed in Figure 2, in which the cumulative frequency of men expressed on a probability scale is plotted against units excreted per hour on a logarithmic scale. Since there is no systematic deviation from a rectilinear relation, the plot indicates that the mean values of all of the excretion results are arranged in the form of a normal distribution curve. Included in Figure 2 is a similar plot of the data ob-

tained from the study of healthy men (2) which reveals that the ulcer group excretes approximately twice as much uropepsin as does the healthy group.

B. Pernicious anemia

It has already been demonstrated by Farnsworth *et al.* (4) that patients with pernicious anemia excrete no uropepsin. In our own studies we have also had the opportunity to perform uropepsin assays on specimens of urine obtained from patients with pernicious anemia. A total of 72 assays was performed on samples of urine collected for from one to 20 consecutive nights from nine patients with pernicious anemia. In every case, no uropepsin was detected. Thus, our own results confirm those of Farnsworth *et al.* (4) in regard to the absence of uropepsin in the urine of such patients.

TABLE III

Uropepsin excretion by patients with gastric complaints without ulcer

Patient	Diagnosis	First four assays					All assays	
		1	2	3	4	Mean	Num-ber	Mean
M. S.	Gastric Ca	.38	.52	.18	.60	.42	10	.49
W. G.	Functional	.48	.23	.71	.51	.48	40	.75
W. B.	Gastric Ca	.54	.52	.04	.51	.40	11	.21
J. C.	Gastric Ca	0	0	.62	.21	.33	3	.21
C. B.	Chronic gastritis	.58	.20	.78	.56	.53	31	.44
H. C.	Spastic duodenitis	.18	.26	0	.62	.27	7	.20
W. K.	Chronic cholecystitis	0	0	0	0	0	4	0
K.	Gastric prolapse	.72	.43	.57	.15	.47	7	.45
E. McQ.	Gastric Ca	.15	.08	.30	.81	.34	11	.39
S.	Epigastric pain etiology unknown	.92	.28	.61	.90	.68	10	.52
T.	Gastric Ca	.68	.69	0	.56	.48	6	.60
G. W.	Hematemesis etiology unknown	.66	.53	0	.52	.43	5	.38
G. F.	Ulcer scar	.84	.64	.88	.73	.77	60	.77
E. S.	Gastric prolapse	1.04	.70	1.00	1.18	.98	42	.66
Mean excretion		.51	.36	.41	.59	.46		.43

C. Gastric complaints, with no demonstrable peptic ulcer

Urine was obtained from a number of patients complaining of ulcer-like and various other complaints referable to the gastrointestinal tract. Such patients included only those in whom no roent-

genologic, gastroscopic or histopathologic evidence of peptic ulcer could be obtained. Included in this group are patients whose clinical symptomology was suggestive of peptic ulcer, patients with carcinomatous lesions of the stomach, and patients with ill-defined gastric complaints.

Uropepsin assays were performed on a total of 247 specimens of urine obtained from 14 such subjects. The results are presented in Table III which demonstrates that most of these patients excreted uropepsin at rates similar to those observed in the group of healthy men. That is, they excreted uropepsin at rates appreciably lower than those characteristic of the uropepsin excretion of patients with proven peptic ulcer.

DISCUSSION

The differences noted in the uropepsin excretion of patients with peptic ulcer as contrasted with that of healthy men and those with other types of diseases, are of great interest. The analysis of variance presented in Table II indicates that a significant difference exists between the ulcer group and the healthy group of subjects. The probability is far less than one in a thousand that the

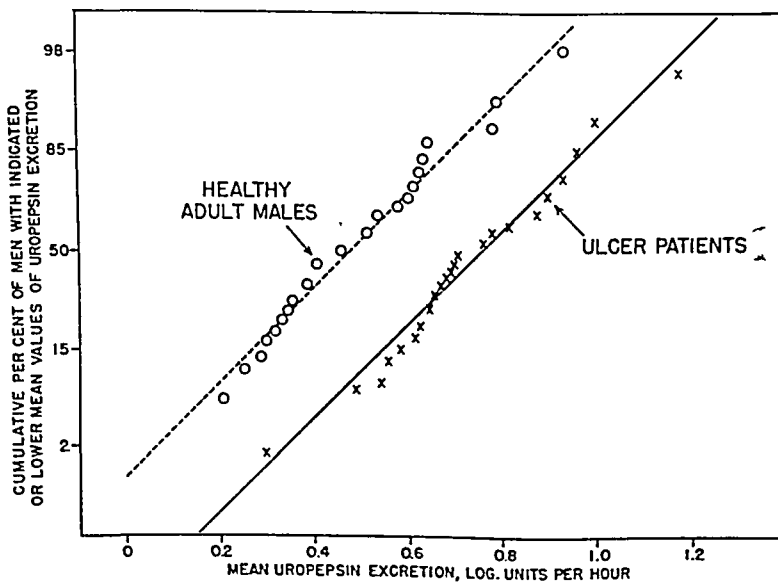


FIG. 2. FREQUENCY DISTRIBUTIONS OF MEAN UROPEPSIN EXCRETIONS OF HEALTHY MEN AND PATIENTS WITH PEPTIC ULCER

Probit plots of the mean uropepsin excretion values of all assays for each individual. The straight lines corresponding to these data have been fitted by inspection. The data for the healthy men have been taken from the previous paper (2).

observed differences in variance could be due to chance grouping of the subjects.

It was also demonstrated by the method of probits that the mean excretion values for all of the subjects in either group (healthy or ulcer) could be arranged in the form of a normal distribution curve (Figure 2). The use of the data in this figure permits a prediction of the type of result that may be anticipated from a study of other ulcer patients or healthy subjects.

As is illustrated in Figure 2, a certain amount of overlapping was found to exist between the uropepsin excretion rates of individuals of the two groups. That the two groups are essentially dissimilar in spite of the observed degree of overlapping was demonstrated by the analysis of variance. Because of the overlapping the rate of uropepsin excretion cannot be utilized to determine whether or not all subjects definitely belong to either the normal or ulcer group.

The success of the prediction as to whether or not an individual belongs to one group or to the other can be visualized through a comparison of the probit plots in Figure 2. These data reveal that the mean value for uropepsin excretion in log units per hour was found to be 0.45 for the healthy subjects, whereas the mean value for the uropepsin excretion of patients with peptic ulcer was found to be 0.74 log units per hour. It can also be seen that, of a group of normal subjects, 98% can be expected to show a mean excretion value of less than 0.88 units. Consequently, any subject with an excretion rate at this or higher levels can be predicted, with relative certainty, to belong outside of the healthy group. On the other hand, 27% of all ulcer patients can be expected to have a mean uropepsin excretion at this or higher levels. Accordingly, any one individual with an excretion rate at this or higher levels, has a much greater chance of belonging to the ulcer than to the normal group.

The probit plots show also that less than 8% of all normal patients will have mean excretion rates of 0.76 log units per hour or more. On the other hand, 50% of all ulcer patients will have a uropepsin excretion rate at, or higher than, this level. Further, 50% of all normal individuals will have uropepsin excretion rates of 0.47 log units per hour or less. On the other hand, less than 8% of ulcer patients can be expected to have mean ex-

cretion rates in this low range. All of these examples indicate the value of the uropepsin excretion determination in predicting whether or not an individual belongs to the normal or ulcer group.

The results in Table I of this report and Table III of the preceding report (2) indicate that a maximum of four consecutive night collections of urine will suffice for the determination of a stable mean uropepsin rate. Although a greater number of assays might yield a somewhat different mean value, the mean excretion value of the group as a whole will not be appreciably changed.

In spite of the limitations indicated by the foregoing discussion, the analysis of variance (Table II) reveals the limits of reliability within which the uropepsin assay can be utilized as an aid in the diagnosis of peptic ulcer. Its greatest usefulness might be found in the differentiation of patients with peptic ulcer from those with various gastric complaints but who do not have a true ulcer since those latter patients resemble healthy subjects in regard to their uropepsin excretion patterns. In view of the simplicity of the uropepsin assay and the lack of inconvenience to the patient, it may be useful in place of the somewhat more difficult and less convenient gastric analysis employed for such purposes. In this connection, it is of interest to note that, on several occasions, the results of the uropepsin assays performed on patients suspected of having ulcer indicated that they did not, in all probability, have such a lesion. This last impression was subsequently confirmed by gastroscopy or by operative findings.

Gottlieb (5) found a definite correlation between the height of uropepsin excretion and the production of acid by the stomach. Thus he noted the highest degree of uropepsin excretion in patients with marked hyperacidity. Since most ulcer patients characteristically display a rather marked hyperacidity, it could be anticipated, according to the observations of Gottlieb, that they would also display an increased uropepsin excretion. However, the correlation noted by Gottlieb between hyperacidity and increased uropepsin excretion may have been fortuitous. At any rate there is ample reason to believe that hyperacidity per se plays no role in regulating the excretion of uropepsin. As was indicated in the preceding reports (1, 2), uropepsin is probably derived from the endocrine secretion of the pepsinogen-produc-

ing cells of the gastric mucosa. A great many studies would indicate a lack of functional interdependence between the pepsinogen-secreting cells and those secreting HCl. Furthermore, our data indicate also that the amount of gastric secretion into the lumen of the stomach may bear no relationship at all to the amount of pepsinogen secreted directly into the blood.

We have had occasion to test the influence of the injection of histamine on uropepsin excretion by man and have been unable to conclude that histamine-induced acid production results in an increase in uropepsin excretion. Such results are in accord with those recently reported by Bucher and Anderson (6) who found that the injection of caffeine and histamine in oil exerted no influence on the uropepsin excretion of the cat although both are known to be powerful gastric stimulants. Furthermore, the results reported in the earlier papers in this series lead to the conclusion that factors such as food, alcohol, etc., which are known to influence the rate of secretion of the gastric juices, exert little influence in determining the rate of uropepsin excretion.

All of this would tend to indicate that pepsinogen is absorbed into the blood stream at a fairly constant rate that is characteristic of each individual, and that acid production by the stomach is not associated in a cause and effect relation with uropepsin excretion. It so happens that patients with peptic ulcer excrete uropepsin at a higher than normal rate and that many such patients also exhibit hyperacidity. Presumably the same factors that increase the endocrine activity of the pepsinogen-secreting glands in the ulcer patient also may operate to increase the rate of secretion of HCl.

The relative degree of constancy of uropepsin excretion by ulcer patients is of some interest. Our earliest impression was to the effect that the uropepsin excretion of ulcer patients was characterized by wider fluctuation (7) than was the case for normal subjects. However, as noted above, the analysis of variance failed to confirm this impression. This would indicate that whatever factors are responsible for the increased rate of uropepsin excretion of ulcer patients operate at a fairly constant strength.

SUMMARY AND CONCLUSIONS

1. Men with peptic ulcer excrete uropepsin at a rate which is approximately twice the rate of healthy men.

2. Previous observations of the absence of uropepsin from the urine of patients with pernicious anemia have been confirmed.

3. Patients with gastric complaints but without peptic ulcer tend to resemble healthy men in regard to their excretion pattern.

4. The data suggest that uropepsin assays may be a useful adjunct in the diagnosis of peptic ulcer. The limits of its value are discussed.

5. The relationship between gastric hyperacidity and uropepsin excretion is discussed. It is concluded that there is no causal relationship between the two factors and that the frequent association of hyperacidity with increased uropepsin excretion is fortuitous and may be ascribed to the influence of unknown factors which may act to increase either acid production by the stomach and/or uropepsin excretion.

ACKNOWLEDGMENT

We wish to acknowledge the critical assistance of Dr. Henry W. Ryder of the Kettering Laboratory of Applied Physiology, who suggested the statistical methods used in the analysis of the data, and the aid rendered by Dr. I. H. Weiland in the collection of samples.

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